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理學博士學位論文

# **CPNE7 and NFI-C mediate odontoblast differentiation and dentin formation**

상아모세포 분화와 상아질 형성 과정에서 CPNE7과  
NFI-C의 역할

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# **ABSTRACT**

## **CPNE7 and NFI-C mediates odontoblast differentiation and dentin formation**

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During the process of tooth morphogenesis, numerous signaling molecules and transcription factors mediate odontoblast differentiation and dentin formation through sequential epithelial-mesenchymal interactions. Dentin is formed by odontoblasts which secrete extracellular matrix (ECM) proteins. Among ECM proteins, dentin sialophosphoprotein (DSPP) is the most abundant ECM in dentin. However, the precise function and molecular mechanisms of odontoblast differentiation are still under investigation.

In the present study, we investigated biological function and mechanisms of Cpne7 and NFI-C in regulation of odontoblast differentiation. Cpne7 was expressed in preameloblasts and inner enamel epithelium. The secreted Cpne7 was translocated to differentiating odontoblasts. In odontoblasts, Cpne7 promoted odontoblastic markers and mineralized nodule formation *in vitro*. Moreover, Cpne7 induces the differentiation of dental pulp cells into odontoblasts and dentin formation *in vivo*. Mechanistically, Cpne7 interacted with Nucleolin and Cpne7-Nucleolin complex promoted the expression of Dspp.

Zinc deficiency is involved in bone malformations and oral disease. Mice deficient in zinc transporter Zip13 show connective tissue and skeletal disorders, abnormal incisor teeth, and reduced root dentin formation in the molar teeth and share a morphologically similar phenotype to nuclear factor I-C (NFI-C)-deficient mice.

In the present study, we determined the NFI-C signaling on odontoblast differentiation and dentin formation with altered zinc concentration. Zinc enhanced the binding efficiency of NFI-C and phosphorylation of Smad2/3 (p-Smad2/3) in the cytoplasm. In zinc deficiency, NFI-C accumulated into nucleus, and NFI-C bound to the Dspp promoter and regulated Dspp gene transcription. Furthermore, zinc deficiency condition promoted DSPP expression in odontoblasts and dentin mineralization, while zinc sufficiency condition decreased DSPP expression and slightly delayed dentin mineralization.

Taken together, Cpne7 and NFI-C signaling regulate odontoblast differentiation and dentin formation through modulation of Dspp gene transcription.

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**Keywords:** CPNE7, NFI-C, odontoblast differentiation, dentin formation

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# **CHAPTER I. GENERAL INTRODUCTION**

## **1. Dentinogenesis**

Tooth morphogenesis is a progressive process regulated by sequential and reciprocal interactions between epithelial and mesenchymal tissues, during which the simple oral ectoderm thickens, buds, grows and folds to form the complex shape of the tooth crown [1].

Dentinogenesis can be described as a complex physiological process of tooth development. Dentin is formed by cells called odontoblasts that differentiate from ectomesenchymal cells of the dental papilla following an organizing influence that emanates from the inner enamel epithelium. During bell stage of tooth development, the differentiation of odontoblasts from the dental papilla in normal development is brought about by the expression of signaling molecules and growth factors in the cells of the inner enamel epithelium. At this time they are separated from the inner enamel epithelium by an acellular zone that contains some fine collagen fibrils. The ectomesenchymal cells adjoining the acellular zone rapidly enlarge and elongate to become preodontoblasts first and then odontoblasts as their cytoplasm increases in volume to contain increasing amounts of protein-synthesizing organelles. The acellular zone between the dental papilla and the inner enamel epithelium gradually is eliminated as the odontoblasts differentiate and increase in size and occupy this zone. These newly differentiated cells are characterized by being highly polarized, with their nuclei positioned away from the inner enamel epithelium. Differentiated odontoblasts secrete

collagenous and noncollagenous matrix proteins (NCPs). Among NCPs, dentin sialophosphoprotein (DSPP) is the most abundant organic matrix protein in dentin. Mineralization of dentin starts from the deposition of minerals in the dentin matrix. Throughout dentinogenesis, mineralization is achieved by continuous deposition of mineral, initially in the matrix vesicle and then at the mineralization front. The cell exerts control in initiating mineralization by producing matrix vesicles and proteins that can regulate mineral deposition and by adapting the organic matrix at the mineralization front so that it can accommodate the mineral deposits [2]. Mutations of the human DSPP gene are associated with dentinogenesis imperfecta type II and type III. Dspp-deficient mice have teeth that display dentin mineralization defects that are similar to those in human dentinogenesis imperfecta type III, indicating that Dspp plays a critical role in odontoblast differentiation and dentinogenesis [3-5].

Predentin consists principally of collagen and is similar to osteoid in bone. Predentin gradually mineralizes into dentin as various noncollagenous matrix proteins are incorporated at the mineralization front. Mature dentin is made up of approximately 70% inorganic materials, 20% organic materials and 10% of water. The inorganic component of dentin consists of substituted hydroxyl apatite in the form of small plates. The organic phase is about 90% collagen with fractional inclusions of various noncollagenous matrix proteins and lipids. The noncollagenous matrix proteins pack the space between collagen fibrils and accumulate along the periphery of dentinal tubules. Dentin is consisted of three types of dentins. Most of the tooth is formed by primary dentin, which outlines the pulp chamber and is referred to as circumpulpal dentin.

Secondary dentin develops after root formation has been completed and represents the continuing, but much slower, deposition of dentin by odontoblasts. Secondary dentin has a tubular structure that, though less regular, is for the most part continuous with that of the primary dentin. Tertiary dentin is produced in reaction to various stimuli, such as attrition, caries, or a restorative dental procedure. Tertiary dentin may have tubules continuous with those of secondary dentin, tubules sparse in number and irregularly arranged, or no tubules at all.

## **2. Epithelial-mesenchymal interaction and CPNE7**

Epithelial mesenchymal interactions (EMIs) are described as a series of programmed, sequential and reciprocal communications between the epithelium and the mesenchyme. In dentinogenesis, these EMIs are a crucial mechanism for development of teeth[6]. Previously, we reported that dental epithelium-derived factors present in the conditioned medium of preameloblasts (PA-CM) induce the odontogenic differentiation of dental mesenchymal cells. Moreover, we identified the protein CPNE7 among those secreted dental epithelium-derived factors, and it has been suggested to be mainly related to odontoblast differentiation [7]. Members of the copine family are ubiquitous calcium dependent phospholipid-binding proteins and the nine copine genes (CPNE1-9) have been identified. They contain two C2 domains (C2Ds) in the N-terminus and a von Willebrand factor type A domain (vWA) in the C-terminus. The C2Ds of copines were originally identified in conventional protein kinase C (PKC) and are involved in calcium influx[8]. The vWA domain mediates protein-protein interaction [9]. Moreover, it has

been demonstrated that copines translocate from cytoplasm into the nucleus (CPNE1, 2,3, and 7) [10], regulate NF-kB gene expression (CPNE1) [11], and interact with the protein nucleolin (CPNE3) [12]. However, there have been few studies about whether Cpne7 can regulate odontoblastic differentiation of mesenchymal cells.

### **3. Nuclear factor I (NFI) family**

The nuclear factor I (NFI) family of site-specific transcription factors, encoded by four genes in vertebrates (termed *Nfia*, *Nfib*, *Nfic*, and *Nfix*), plays essential developmental roles in the transcriptional modulation of various cell types. NFI family of proteins displays the unusual property of regulating not only the initiation of transcription but also of mediating DNA replication. NFI recognition sequences were found in the promoter sequences of many cellular genes, where they may act as activator or repressor of transcription. NFI proteins contain a highly conserved NH<sub>2</sub>-terminal domain that mediates DNA binding and a dimerization domain is gene-specific [13, 14].

Disruption of the *Nfic* gene in mice leads to abnormal tooth roots that are predominantly caused by abnormal odontoblast differentiation and dentin formation during root formation [15, 16].

### **4. Zinc homeostasis**

Zinc is an essential trace element for growth, development, and health maintenance. Zinc is found in nearly 300 specific enzymes, serves as structural ions in transcription factors and other biological functions. In humans, zinc plays ubiquitous biological roles

in the metabolism of RNA and DNA, signal transduction, and gene expression. It also regulates apoptosis [17, 18]. Moreover, Zinc deficiency is usually due to insufficient dietary intake, but can be associated with malformation and retardation of bone growth, impairment of immune responses, chronic liver disease, diabetes, malignancy, and other chronic illnesses [19-22]. Consequently, intracellular zinc concentrations are extremely important for the maintenance of regulatory systems.

The zinc transporter Slc39a13/Zip13 influences intracellular zinc levels by transporting zinc from the Golgi to the cytosol. Zinc transporter Zip13 deficient mice show connective tissue and skeletal disorders, abnormal incisor teeth, and reduced root dentin formation in the molar teeth [23]. Interestingly, these mice share a morphologically similar phenotype to Nfic-deficient mice. However, the precise mechanism by which zinc regulates the NFI-C gene for odontoblast differentiation and dentin formation remains unknown.

## **5. Rationale and outline of the thesis experiments**

A key purpose of this thesis is to investigate the mechanisms of Cpne7 and NFI-C in dentinogenesis. We investigated biological function and mechanism of Cpne7 in regulation of dental and non-dental mesenchymal cell differentiation into odontoblasts via epithelial-mesenchymal interaction. And we conducted to assess how NFI-C signaling and zinc concentration changes affect odontoblast differentiation and mineralization. We also determined whether zinc equilibrium is required for dentin formation through the nuclear accumulation and modulation of NFI-C.

## **CHAPTER II.**

# **CPNE7, a preameloblast-derived factor, regulates odontoblastic differentiation of mesenchymal stem cells**

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## **I. ABSTRACT**

Tooth development involves sequential interactions between dental epithelial and mesenchymal cells. Our previous studies demonstrated that preameloblast-conditioned medium (PA-CM) induces the odontogenic differentiation of human dental pulp cells (hDPCs), and the novel protein Cpne7 in PA-CM was suggested as a candidate signaling molecule. In the present study, we investigated biological function and mechanisms of Cpne7 in regulation of odontoblast differentiation. Cpne7 was expressed in preameloblasts and secreted extra cellularly during ameloblast differentiation. After secretion, Cpne7 protein was translocated to differentiating odontoblasts. In odontoblasts, Cpne7 promoted odontoblastic markers and the expression of Dspp in vitro. Cpne7 also induced odontoblast differentiation and promoted dentin/pulp-like tissue formation in hDPCs in vivo. Moreover, Cpne7 induced differentiation into odontoblasts of non-dental mesenchymal stem cells in vitro, and promoted formation of dentin-like tissues including the structure of dentinal tubules in vivo. Mechanistically, Cpne7 interacted with Nucleolin and modulated odontoblast differentiation via the control of Dspp expression. These results suggest Cpne7 is a diffusible signaling molecule that is secreted by preameloblasts, and regulates the differentiation of mesenchymal cells of dental or non-dental origin into odontoblasts.



## II. INTRODUCTION

During tooth morphogenesis, numerous signaling molecules and transcription factors mediate odontoblast differentiation and dentin formation through sequential epithelial-mesenchymal interactions [1]. Dentin is formed by odontoblasts, which are derived from dental papilla ectomesenchymal cells. Odontoblast differentiation is induced by signaling molecules and growth factors derived from inner enamel epithelial cells, which are located directly adjacent to the dental papilla [2]. After the epithelial induction, odontoblasts secrete the organic matrix proteins, which consist mainly of type I collagen, dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1), and are then modified in order to initiate mineralization [3-5]. However, the precise molecular mechanisms of odontoblast differentiation mediated through epithelial-mesenchymal interaction are still under investigation.

Previously, we reported that dental epithelium-derived factors present in the conditioned medium of preameloblasts (PA-CM) induce the odontogenic differentiation of dental mesenchymal cells, such as dental pulp stem cells, and promote dentin formation in vitro and in vivo. Moreover, we identified the protein Cpne7 among those secreted dental epithelium-derived factors, and it has been suggested to be mainly related to odontoblast differentiation [7]. Members of the copine family are ubiquitous calcium dependent phospholipid-binding proteins and are evolutionally conserved from *Arabidopsis* to *Homo sapiens*. The nine copine genes (CPNE1-9) have been identified. They contain two C2 domains (C2Ds) in the N-terminus and a von Willebrand factor type A domain (vWA) in the C-terminus. The C2Ds of copines were originally identified in conventional protein

kinase C (PKC) and are involved in calcium influx [8]. The vWA domain mediates protein-protein interaction [9]. Moreover, it has been demonstrated that copines translocate from cytoplasm into the nucleus (CPNE1, 2,3, and 7) [10], regulate NF- $\kappa$ B gene expression (CPNE1) [11], and interact with the protein nucleolin (CPNE3) [12]. However, there have been few studies about whether Cpne7 can regulate odontoblastic differentiation of mesenchymal cells.

In the present study, we investigated biological function and mechanisms of Cpne7 in regulation of dental and non-dental mesenchymal cell differentiation into odontoblasts via epithelial-mesenchymal interaction. Our findings suggest the novel concept that Cpne7, a dental epithelium-derived protein, plays an essential role in commitment of odontoblasts, odontoblast differentiation, and dentin formation.

### **III. MATERIALS AND METHODS**

#### **1. Co-culture system**

MDPC-23 cells were provided by Dr. J.E. Nor (University of Michigan, Ann Arbor, MI). ALCs were provided by Dr. T. Sugiyama (Akita University School of Medicine, Akita, Japan). For co-culture of ALCs with MDPC-23 cells, Transwell® permeable supports (Corning Inc., Corning, NY) were used. The ALCs were seeded in the upper compartment of the transwell, and the MDPC-23 cells were seeded in the lower compartment of the dish. When cells reached 80%-90% confluence, the upper compartment was combined with the lower compartment.

#### **2. Cell culture**

C3H10T1/2 and HEK293 human embryonic kidney cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human BMSCs (PT-2501) were purchased from Lonza (Walkersville, MD). MDPC-23, C3H10T1/2, hBMSCs, and HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Grand Island, NY), and ALCs were cultured in minimum essential medium (MEM; Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) and antibiotic-antimycotic (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO<sub>2</sub>. To induce cell differentiation and mineralized nodule formation, confluent cells were treated with induction medium (DMEM containing 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate) for up to 2 weeks.

### **3. Plasmids**

Full-length mouse Cpne7 (mCpne7, NM\_170684) cDNA, siRNA targeting Cpne7, and pGL3-Dspp vectors were constructed and verified as described previously [7]. Expression vectors encoding full-length human CPNE7, green fluorescent protein (GFP)-CPNE7 (NM\_153636), DDK (Flag)-tagged CPNE7 (NM\_153636) and Recombinant CPNE7 (NP 705900) were purchased from Origene (Rockville, MD). Control siRNA (sc-37007) and siRNA targeting nucleolin (sc-29230) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

### **4. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analysis**

Total RNA was extracted from the cells with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (3 mg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (New England Biolabs, Ipswich, MA). One microliter of the RT product was amplified by PCR using the following primer pairs: Odam (462 bp, forward [F] 50-atgtcctatgtggttcctgt-30, reverse [R] 50-ttatggttctcttaggctatc-30), Cpne7 (178 bp, [F] 50-cccgacccattgaccaagtc-30, [R] 50-catacacctcaaaccgtagcttc-30 ), Mmp-20 (458 bp, [F] 50-agctgtgagcaactgatgactgga-30 , [R] 50-acagctagagccaagaacacacct-30), Gapdh (452 bp, [F] 50-accacagtccatgccatcac-30 , [R] 50-tccaccaccctgttgctgt 30), Dspp (141 bp, [F] 50-gtgaggacaaggacgaatctga-30 , [R] 50-cactactgtcactgtgtcact-30 ), and Osteocalcin (283 bp, [F] 50-ccacagccttcattgtccaag-30 , [R] 50-ggcagagagagaggacaggg-30). PCR was carried out under conditions of 30 cycles of

94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA) using SYBR Green PCR Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were 40 cycles of 95 °C for 1 min, 94 °C for 15 s, and 60 °C for 34 s. All reactions were run in triplicate, and PCR product levels were normalized to that of the housekeeping gene Gapdh. Relative changes in gene expression were calculated using the comparative threshold cycle (CT) method.

## **5. Preparation of CM and inactivation of Cpne7**

CM was harvested as described previously [7]. Briefly, ALC cells were seeded at  $7.5 \times 10^5$  cells/100 mM dish. When the cells reached 80% confluence, medium was replaced with differentiation medium. After 3 days of differentiation, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in differentiation medium without FBS for 24 h. The serum-free conditioned medium was harvested and concentrated to 1 mg/ml by ammonium sulfate precipitation. To inactivate Cpne7 in CM, 20 or 40 mg/ml anti-Cpne7 antibodies was mixed with CM for 2 h at 4°C with rotation.

## **6. Western blot analysis**

Western blot analyses were performed as previously described [7]. Briefly, proteins (30 mg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Rabbit and affinity-purified polyclonal anti-Odam, anti-Cpne7, and anti-Dsp antibodies were produced as described previously [7, 24]. The Flag (F3165) antibody was purchased from Sigmae Aldrich (St. Louis, MO). The Mmp-20 (sc-26926), Lamin B (sc-6216), Osteocalcin (sc-30044), Nucleolin (sc-8031), and Gapdh (sc-25778) antibodies were purchased from Santa Cruz Biotechnology. The Dmp1 antibody (ab103203) was purchased from Abcam (Cambridge, UK). The labeled protein bands were detected using an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK).

## **7. Transient transfection and luciferase assay**

HEK293, MDPC-23 or C3H10T1/2 cells were seeded in 12-well culture plates at a density of  $1.5 \times 10^5$  cells per well. The cells were transiently transfected using Metafectene Pro reagent (Biontex, Martinsried/Planegg, Germany) with the constructs as described above. Construct pGL3-Dspp was co-transfected into MDPC-23 cells with the Cpne7 expression vector or the Cpne7 or Nucleolin siRNA vector. Construct pGL3-Bsp was transfected into C3H10T1/2 cells with rCpne7 treatment. Following the addition of 50 ml luciferin to 50 ml of the cell lysate, luciferase activity was determined using an analytical luminescence luminometer (Promega, Madison, WI) according to the manufacturer's instructions.

## **8. Co-IP assay**

After transfection with the indicated plasmid DNA using Metafectene Pro reagent, MDPC-23 cells were washed in PBS, and the cell lysates were prepared by adding 1 ml of co-IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA) supplemented with protease inhibitors. The lysates were incubated at 4 °C for 2 h with a 1:200 dilution of antibodies. Thirty microliters of protein A/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology) were added and incubated at 4 °C for 1 h with rotation. The immune complexes were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. Following electrophoresis on 10% SDS-PAGE gels, the immunoprecipitates were analyzed by western blotting with anti-Nucleolin.

## **9. ChIP assay**

ChIP assays were performed as previously described [25]. Briefly, after transfection with the indicated plasmid DNA using Metafectene Pro reagent, MDPC-23 cells were sonicated. The fragmented chromatin mixture was incubated with anti-Nfic, anti-Nucleolin, anti-Cpne7, or IgG (1:250) on a rotator at 4 °C for 4 h. Thirty microliters of protein A/G PLUS-agarose were added and incubated at 4 °C for 1 h with rotation to collect the antibody/chromatin complexes. The final DNA pellets were recovered and analyzed by PCR using primers that encompass the Dspp promoter region (390 bp), mouse Dspp-400 region: forward 50-gggtcttaaatagccagtcg-30 and mouse Dspp-10 region: reverse, 50-ctgagagtggcacactgt-30. PCR was carried out under conditions of 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR products were electrophoresed

in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

## **10. Animals, tissue preparation, and immunohistochemistry**

All experiments using animals followed protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-111013-2). The heads of mice at embryonic day 19 (E19), postnatal day 7 (P7) and day 10 (P10) were decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and processed for immunohistochemistry. Cpne7 expression was detected using an ABC kit (Vector Labs, Burlingame, CA) with rabbit anti-Cpne7 as the primary antibody and a biotin-labeled goat anti-rabbit IgG (1:200, Vector Labs) as the secondary antibody.

## **11. Primary cell culture, in vivo transplantation, and histological analysis**

We collected human impacted third molars at Seoul National University Dental Hospital (Seoul, Korea). The experimental protocol was approved by the Institutional Review Board. Informed consent was obtained from the patients. Human DPCs were isolated and used in an in vivo transplantation experiment as described previously [7]. The hDPCs ( $2 \times 10^6$ ) were mixed with 100 mg hydroxyapatite/tricalciumphosphate (HA/TCP) ceramic powder (Zimmer, Warsaw, IN) alone, or with CM (30 mg), or with Cpne7 antibody-treated CM, or with rCpne7 (5 mg) in an 0.5% fibrin gel, and then transplanted subcutaneously into immunocompromised mice (NIHbg-nu-xid; Harlan Laboratories, Indianapolis, IN) for 6 weeks. To inactivate Cpne7 in CM, Cpne7 antibody (5 ml) was mixed with CM for 24 h at 4 °C with rotation before in vivo transplantation in the Cpne7



antibody-treated CM group. For histomorphometric analysis of newly formed mineralized tissue, hDPCs transfected with the Cpne7 overexpression or inactivation constructs were transplanted with HA/TCP particles for 12 weeks, as described above. To evaluate dentin/pulp-like tissue formation in root canal spaces, hDPCs ( $2 \times 10^6$ ) were mixed with CM (10 mg) or Cpne7 antibody-treated CM in a 0.5% fibrin gel, and inserted into the empty root canal spaces of the human tooth segments for 12 weeks. Human tooth segments were fabricated according to the protocol described in our previous study [26]. In addition, hBMSCs were also inserted into the empty root canal spaces with rCpne7 (5 mg) or rBmp2 (5 mg; Cowellmedi, Busan, Korea) for 6 weeks.

Samples were harvested and fixed in 4% paraformaldehyde, decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and stained with hematoxylin-eosin (H-E) (Vector Labs) or processed for immunohistochemistry. For immunohistochemistry, proteins were detected with anti-DSP, anti-BSP [7], or anti-NESTIN (MAB353, Millipore) at a dilution of 1:100 as the primary antibody and a biotin-labeled goat anti rabbit IgG (Vector Labs) as the secondary antibody. The total mineralized area among the regenerated pulp- and dentin-like tissue was analyzed using the LS starter program (Olympus Soft Imaging Solutions, Münster, Germany).

## **12. Fluorescence Microscopy**

The cells in Nunc™ Lab-Tek™ II chambered cover glasses (Thermo Scientific, Waltham, MA) were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized for 10 min in PBS containing 0.5% Triton X-100.

After washing, the cells were incubated with anti-Cpne7 (1:200) and anti-Nucleolin (1:200) antibodies in blocking buffer (PBS and 1% bovine serum albumin) for 1 h followed by the addition of a fluorescein isothiocyanate (FITC)- or Cy3-conjugated anti-mouse or anti-rabbit IgG antibody (1:200, GE Healthcare). After washing, the cells were visualized using an Olympus AX70 fluorescence microscope (Tokyo, Japan). Chromosomal DNA in the nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO).

### **13. Alizarin Red S Staining**

MDPC-23 cells were fixed with 4% paraformaldehyde in PBS for 20 min. The cells were stained with a 1% alizarin red S (Sigma-Aldrich) solution in 0.1% NH<sub>4</sub>OH (pH 4.2) for 20 min at room temperature.

### **14. DAPA**

The binding assay was performed by mixing 600 µg of nuclear extract protein, 6 µg of biotinylated, specific wild-type and mutated AP-1 site oligo nucleotides of the *Dspp* promoter, and 60 µl of streptavidin-agarose beads. The mixture was incubated at 4°C for 2 h with rotation. Beads were pelleted and washed three times with PBS. Bound proteins were eluted with loading buffer, separated by SDS-PAGE, and analyzed by western blotting.

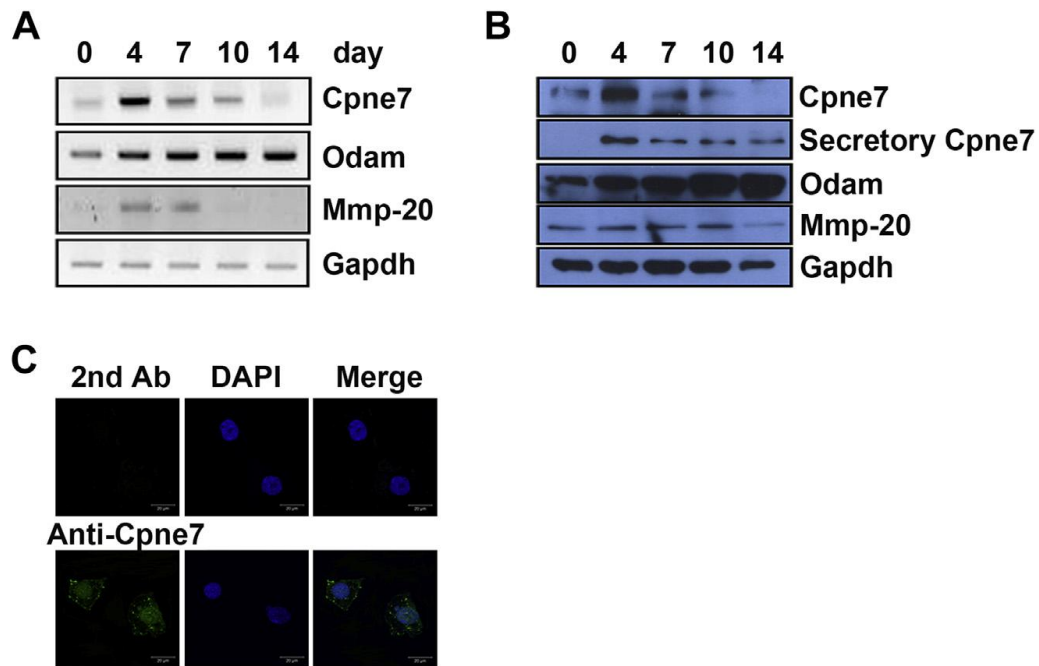
## **15. Statistical analyses**

Statistical analyses were carried out using a Student's t-test. All statistical analyses were performed using SPSS software ver. 19.0.

## **IV. RESULTS**

### **1. Preameloblasts secrete Cpne7 during ameloblast differentiation**

The copine family has been shown to be expressed in various mammalian tissues, including brain, heart, lung, liver, and kidney [9]. Previously, Cpne7 was identified among the secretory proteins of preameloblasts [7]. In our first series of experiments, we cultured ameloblast lineage cells (ALCs) for 2 weeks and analyzed Cpne7 expression by RT-PCR and western blotting to examine the expression patterns of Cpne7 mRNA and protein during ameloblast differentiation. Cpne7 was expressed from day 0 in culture, increased on day 4 (early stage of ameloblast differentiation; preameloblast stage), and then declined gradually from days 7-14 (late stage of ameloblast differentiation and mineralization). Odam and Mmp-20, ameloblast differentiation markers, were expressed during ameloblast differentiation (Fig.1A and B), as expected [24]. Furthermore, secretory Cpne7 was clearly detected in ameloblast conditioned medium (CM) from day 4 (preameloblast stage) and then decreased thereafter (Fig. 1B). The subcellular localization of Cpne7 was in cytoplasm and nucleus in ALCs (Fig. 1C). These results indicate that Cpne7 is secreted into extracellular matrix soon after synthesis by differentiating preameloblasts.

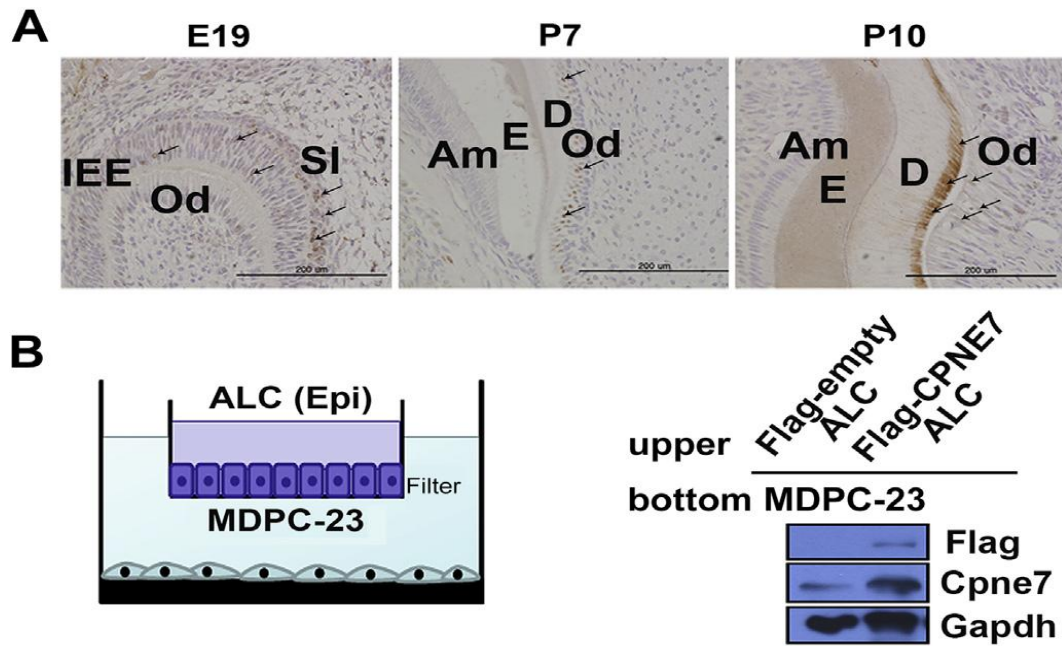


**Fig. 1. Cpne7 is expressed during ameloblast differentiation.**

The ameloblast lineage cell (ALC) line was cultured in differentiation medium for up to 2 weeks. (A) The expression of Cpne7, Odam, and Mmp-20 mRNA in ALCs was evaluated by RT-PCR. (B) The expression of Cpne7, Odam, and Mmp-20 in ALCs on the indicated days was evaluated by western blot analysis. Cpne7, Odam and Mmp-20 were detected in whole cell lysates. Secretory Cpne7 was detected in ALC-CM. (C) Localization of Cpne7 in ALCs was examined by immunofluorescence. Scale bars, 20  $\mu$ m.

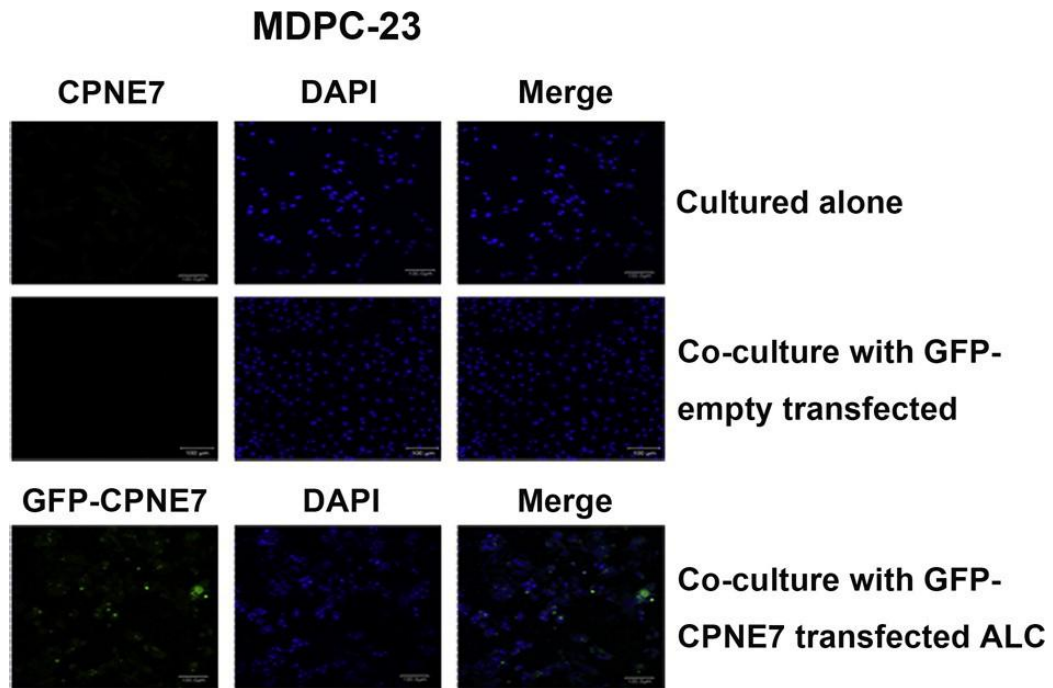
## **2. Cpne7 is translocated from preameloblasts to differentiating odontoblasts**

Paracrine signaling is a form of cell communication system whereby signaling molecules are released by cells and act on neighboring cells [27]. During dentinogenesis, coordinated functions between inner enamel epithelium and dental papilla cells are achieved by paracrine signaling via epithelial-mesenchymal interaction[28]. First, we used immunohistochemistry to investigate localization of Cpne7 protein during tooth development and possibility of Cpne7 as a paracrine signaling molecule. At embryonic day 19 (E19), Cpne7 was expressed in inner enamel epithelium and stratum intermedium of the developing mouse mandibular first molar which was at the bell stage of development. At postnatal day 7 (P7), Cpne7 was localized in differentiating odontoblasts; however, Cpne7 was no longer detected in ameloblasts. Subsequently at P10, Cpne7 was clearly detected in the odontoblasts, pre-dentin, and dentinal tubules (Fig. 2A). The expression of Cpne7 showed a specific temporospatial distribution pattern in mouse molar during dentinogenesis, indicating the translocation of Cpne7 from Preameloblasts to differentiating odontoblasts *in vivo*. To confirm the translocation of secretory Cpne7 from preameloblasts, ALCs in which Flag-tagged CPNE7 was overexpressed were co-cultured with odontoblastic MDPC-23 cells *in vitro*. Flag-tagged CPNE7 was detected in MDPC-23 cells by western blotting (Fig. 2B). Moreover, exogenous Cpne7 was seen in MDPC-23 cells by confocal microscopy (Supporting Information Fig. S1). These findings suggest that Cpne7 secreted by preameloblasts is translocated to differentiating odontoblasts in response to epithelial-mesenchymal interaction.



**Fig. 2. Cpne7 is translocated from ameloblast to odontoblasts.**

(A) Cpne7 expression was detected by immunohistochemistry during mouse tooth development on embryonic day 19 (E19) and postnatal days 7 (P7) and 10 (P10). Cpne7 protein was localized in the inner enamel epithelium and stratum intermedium at E19; in differentiating odontoblasts at P7; and in dentin, pre-dentin, and differentiated odontoblasts at P10 (arrows). IEE: inner enamel epithelium, SI: stratum intermedium, Od: odontoblast, Am: ameloblasts, E: enamel, D: dentin. Scale bars, 200  $\mu$ m. (B) Schematic diagram of the co-culture system. ALCs transfected with Flag-tagged CPNE7 were seeded in the upper chamber and odontoblastic MDPC-23 cells were seeded in the bottom chamber. Epi: epithelial cells, Mes: mesenchymal cells. Translocation of Flag-tagged CPNE7 synthesized by ALCs to MDPC-23 cells in the co-culture system was detected by western blotting.



**Fig. S1. Cpne7 is translocated from ALCs to MDPC-23 cells.**

ALCs transfected with GFP-tagged CPNE7 were seeded in the upper chamber, and MDPC-23 cells were seeded in the bottom chamber. Migration of GFP-tagged CPNE7 from ALCs into MDPC-23 cells was detected by fluorescence microscopy. Cells were counterstained with DAPI. Scale bars, 100  $\mu$ m



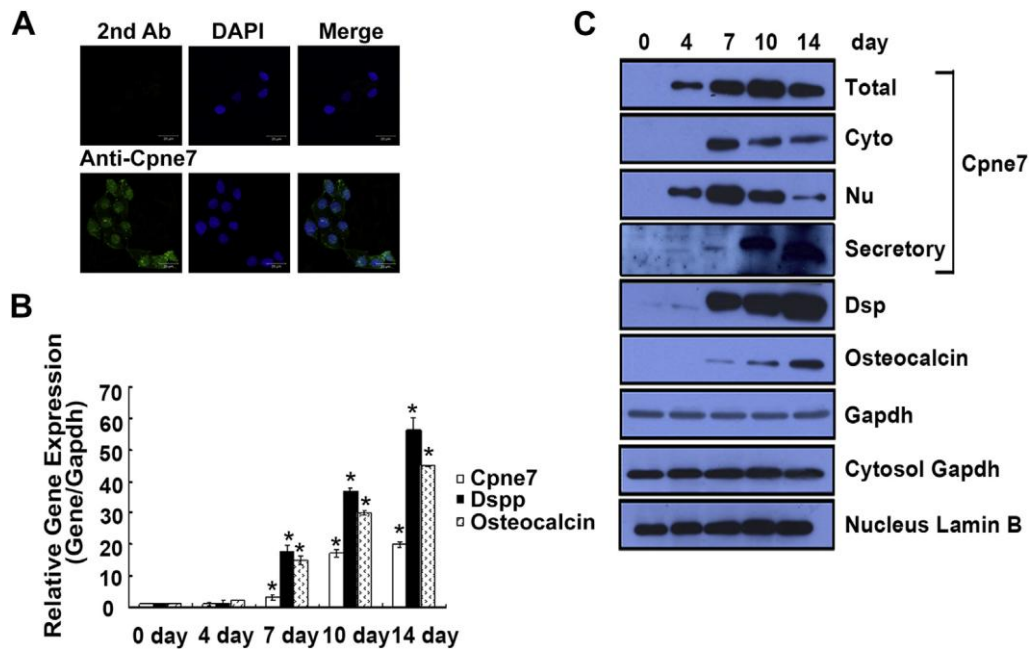
### **3. Cpne7 in differentiating odontoblasts regulates their differentiation into mature odontoblasts in vitro**

A spontaneously immortalized and cloned cell line MDPC-23 has been derived from dental papilla of 18-19 fetal day CD-1 mouse, which already induced by underlying inner enamel epithelium [29]. Prior to clarifying the functional role of translocated Cpne7 in differentiating odontoblasts, the expression pattern of endogenous odontoblast Cpne7 was evaluated in odontoblastic MDPC-23 cells. Cpne7 was localized mainly in nuclei of MDPC-23 cells (Fig. 3A). The expression levels of Cpne7 protein in differentiating odontoblasts increased from days 0-7 (early to middle stage of odontoblast differentiation), and then decreased from days 10-14 (late stage of odontoblast differentiation and mineralization). The expression of odontoblast differentiation markers, including Dsp and Osteocalcin, increased in odontoblasts from days 7-14, which was later than the increase in Cpne7. Interestingly, secretory Cpne7 was clearly detected in odontoblast conditioned medium from days 10-14, whereas nuclear Cpne7 was mainly localized in differentiating odontoblasts from days 4-10 (Fig. 3B and C). These findings provide significant evidences that nuclear Cpne7 in odontoblasts might have functional roles in their differentiation, especially in early to middle stages, and also that the existence of autocrine/paracrine Cpne7 in differentiating odontoblasts of late stage.

To investigate whether Cpne7 influences odontoblast differentiation by upregulating Dspp, we measured the expression levels of Dspp mRNA and Dsp protein [30], after transfection of MDPC-23 cells with constructs encoding Cpne7 and Cpne7 siRNA. The expression level of Cpne7 was increased by transfection with Cpne7-encoding construct

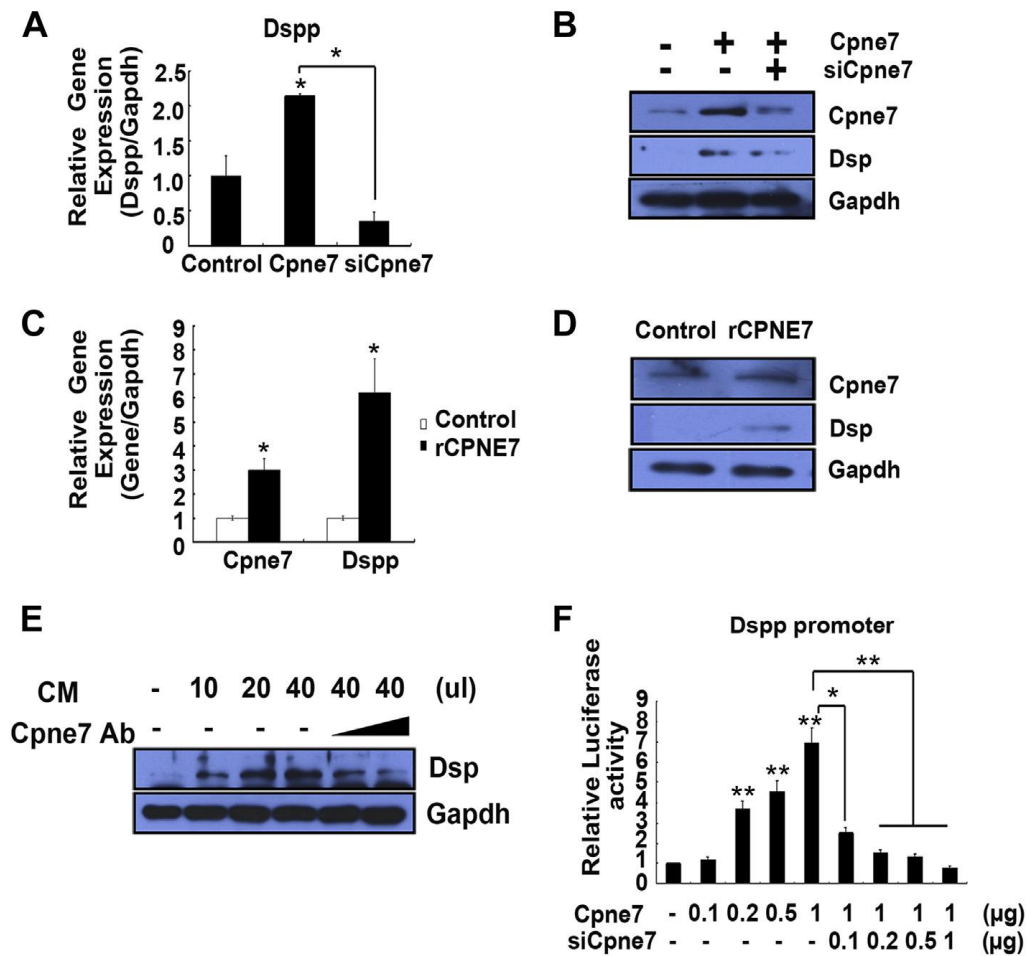
and effectively inhibited by Cpne7 siRNA in MDPC-23 and HEK293 cells, compared with control and cells transfected with control siRNA (Supporting Information Fig. S2, 4B). Cpne7 overexpression significantly increased the expression levels of Dspp mRNA and Dsp protein, whereas siRNA mediated Cpne7 knockdown downregulated them in MDPC-23 cells (Fig. 4A and B). To confirm whether exogenous Cpne7 also enhanced Dspp expression, we investigated the effects of recombinant CPNE7 (rCPNE7) on Dspp expression in MDPC-23 cells. As expected, rCPNE7 treatment also increased the expression levels of Dspp mRNA and Dsp protein in MDPC-23 cells (Fig. 4C and D). Recombinant Cpne7 treatment enhanced mineralized nodule formation compared to control during odontoblast differentiation in vitro (Supporting Information Fig. S3). These data suggest that Cpne7 regulate Dspp expression and control odontoblast differentiation and mineralization.

Next approach was taken to confirm the functional consequences of Cpne7 and Dspp gene expression. The Dsp protein was increased in a dose-dependent manner by treatment with PA-CM, but decreased by treatment with increasing concentrations of Cpne7-specific antibody for inactivation (Fig. 4E). Moreover, the Dspp transcriptional activity was significantly promoted by overexpression of Cpne7, but suppressed by siRNA-mediated Cpne7 knockdown (Fig. 4F). Collectively, these results indicate that Cpne7 regulates odontoblast differentiation and mineralization via control of Dspp expression.



**Fig. 3. Cpne7 is expressed during odontoblast differentiation.**

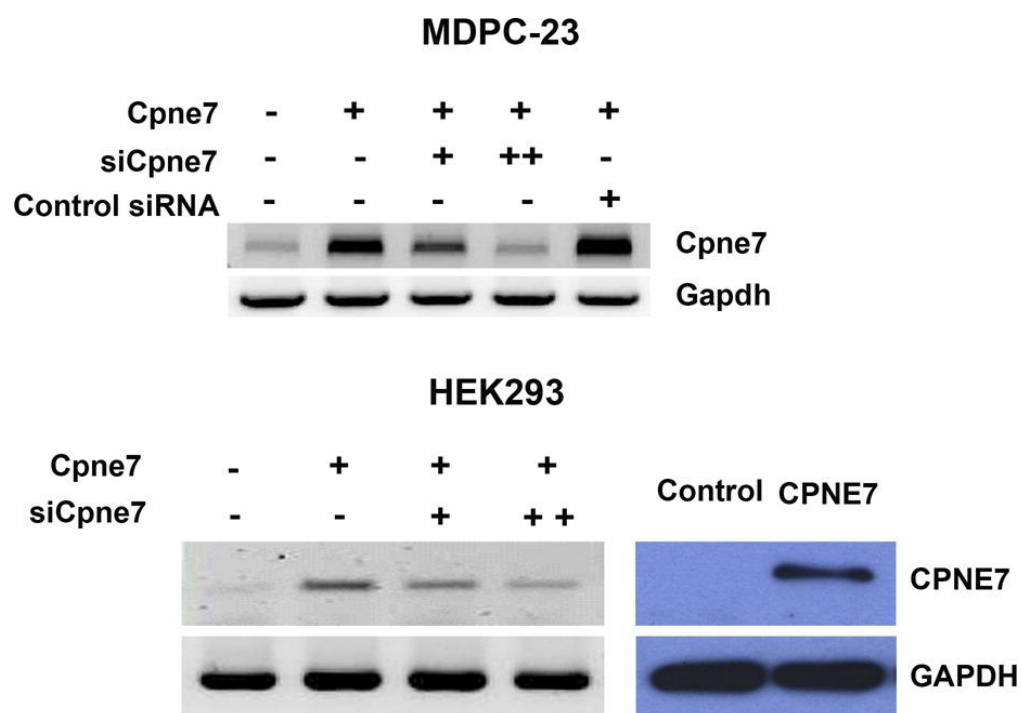
The odontoblast cell line MDPC-23 was cultured in differentiation medium for up to 2 weeks. (A) Localization of Cpne7 in MDPC-23 cells was detected by immunofluorescence. Scale bars, 20  $\mu$ m. (B) Levels of Cpne7, Dspp, and Osteocalcin mRNA were evaluated by quantitative real-time PCR on the indicated days in MDPC-23 cells. All values represent the mean  $\pm$  standard deviation (SD) of triplicate experiments. \* $P < .01$  compared to control. (C) Cpne7, Dsp, and Osteocalcin levels in MDPC-23 cells on the indicated days were evaluated by western blot. Cytosolic Gapdh and nuclear Lamin B served as cell fractionation controls. Total: whole cell extract, Cyto: cytosolic fraction, Nu: nuclear fraction.



**Fig. 4. Cpne7 stimulation and inactivation regulates odontoblast differentiation.**

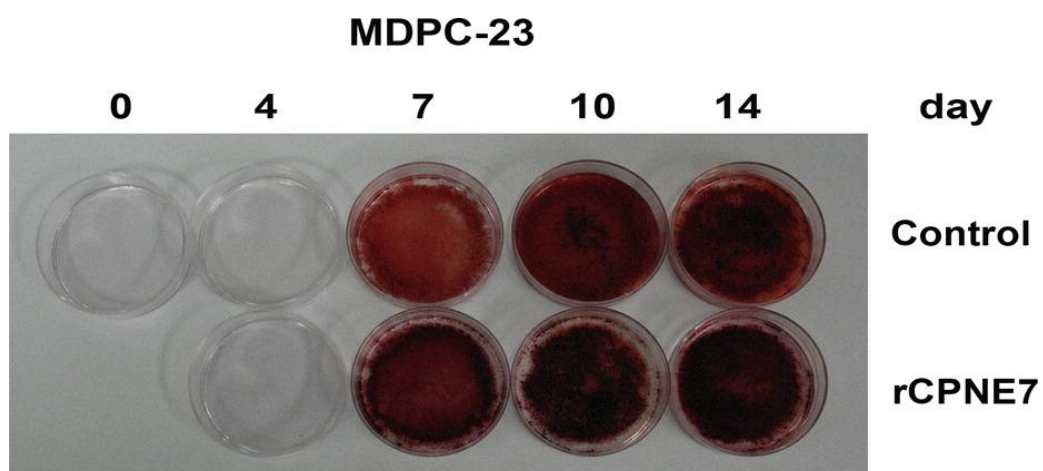
(A, B) MDPC-23 cells were transfected with Cpne7 overexpression or siRNA construct. (A) Expression of Dspp mRNA was analyzed by real-time PCR. (B) Expression of Cpne7 and Dsp was analyzed by western blotting. (C, D) MDPC-23 cells were treated with 100 ng/ml rCPNE7. (C) Expression of Cpne7 and Dspp mRNA was evaluated by real-time PCR. (D) Expression of Cpne7 and Dsp protein was evaluated by western blotting. (E) Expression of Dsp in MDPC-23 cells after inactivation of Cpne7 in CM analyzed by

western blotting. (F) Transcriptional activity of Dspp promoter was evaluated by luciferase assay using overexpression of Cpne7 or Cpne7 siRNA in MDPC-23 cells. All values represent the mean  $\pm$  SD of triplicate experiments. \*P < .05, \*\*P < .01 compared to control.



**Fig. S2. Expression of Cpne7 is regulated by Cpne7 overexpression and Cpne7 siRNA in HEK 293 and MDPC-23 cells.**

Constructs encoding Cpne7, Cpne7 siRNA, and control siRNA were transfected into HEK293 and MDPC-23 cells. (Upper panel) Cpne7 expression in MDPC-23 cells was analyzed by RT-PCR. (Lower panel) CPNE7 expression in HEK293 cells was analyzed by RT-PCR and western blotting.



**Fig. S3. Effects of rCPNE7 on mineralized nodule formation *in vitro***

MDPC-23 cells were cultured for 2 weeks with or without rCPNE7 treatment. The effect of rCPNE7 on mineralized nodule formation was analyzed by alizarin red S staining on the indicated days.

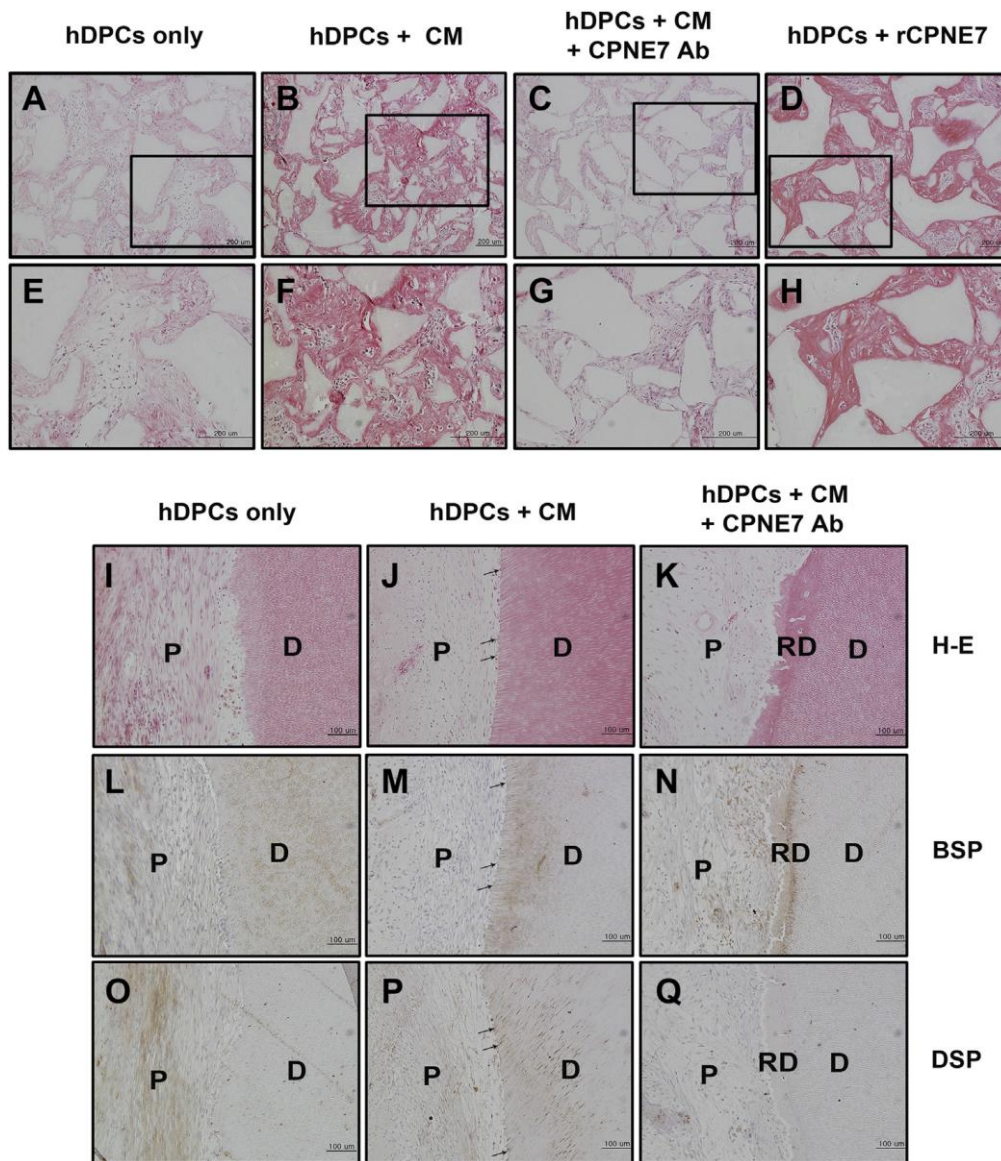
#### **4. Cpne7 induces the differentiation of dental mesenchymal cells, such as human dental pulp cells (hDPCs), into odontoblasts and dentin formation in vivo**

To determine the role of Cpne7 in odontoblast differentiation and dentin formation in vivo, we transplanted hDPCs into the subcutaneous tissues of immunocompromised mice in the presence of hydroxyapatite/tricalcium phosphate (HA/TCP) under four different conditions, hDPCs-only, hDPCs with PA-CM, hDPCs with PA-CM and Cpne7 antibody, and hDPCs with rCPNE7. Six weeks after transplantation, dentin/pulp-like tissues were formed at the periphery of HA/TCP particles in all groups (Fig. 5A-H). PA-CM- or rCPNE7-treated groups exhibited dentin/pulp complex characteristics with odontoblasts more typical than the hDPCs-only group (Fig. 5E, F and H). However, in the group treated with Cpne7 antibody-treated PA-CM for inactivation, mineralized tissue formation was barely observed (Fig. 5F and G).

Based on the results of in vitro experiments shown in Fig. 4, we transplanted hDPCs transfected with Cpne7-encoding, Cpne7 siRNA, or control siRNA constructs into subcutaneous tissue in order to evaluate the effects of the Cpne7 gene on dentin/pulp-like tissue formation in vivo. Twelve weeks after transplantation, hDPCs-only, Cpne7 overexpression, and control siRNA groups showed the generation of dentin-like mineralized tissues at the periphery of HA/TCP particles, whereas the Cpne7 siRNA group revealed little evidence of mineralization (Supporting Information Fig. S4A-H). According to the histomorphometric analysis of each group, overexpression of Cpne7 in hDPCs was associated with the highest level of dentin-like mineralized tissue formation



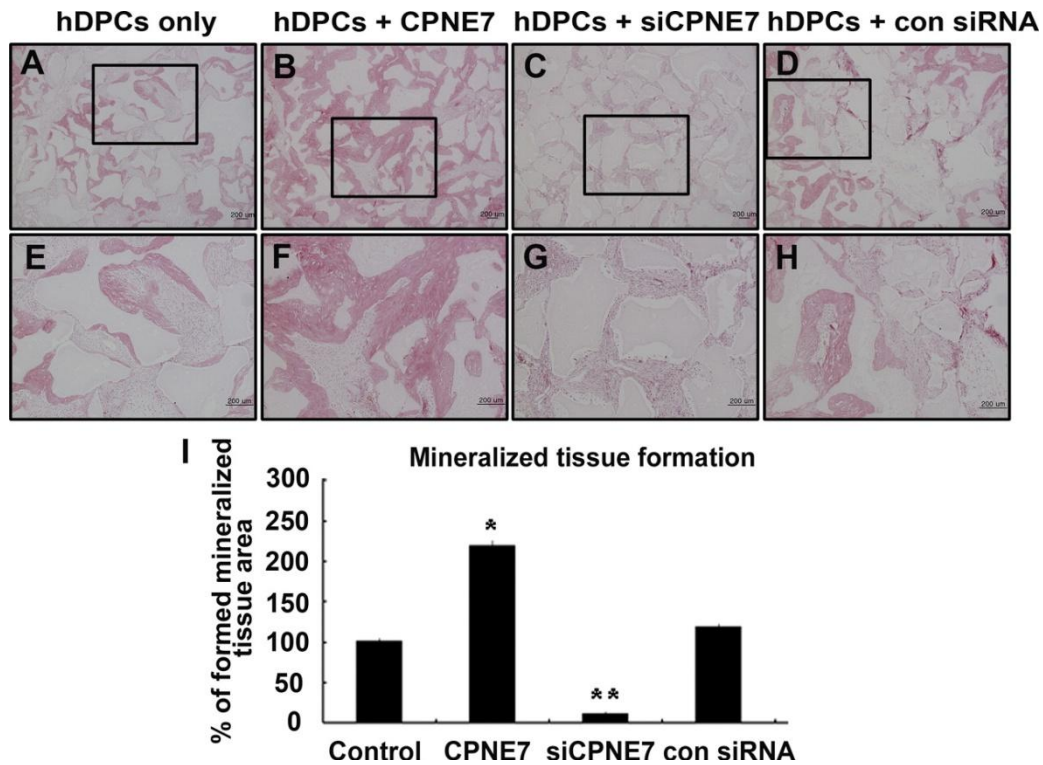
in vivo and siRNA-mediated knockdown of Cpne7 was associated with the least. There was no significant difference between the hDPCs-only and control siRNA groups (Supporting Information Fig. S4I). The tooth segments, which consist of natural dentinal wall and empty pulp cavity, provide the specific local environment for the regeneration of dentin/pulp-like tissues by dental stem cells [31]. To evaluate dentin/pulp-like tissue formation in root canal spaces, hDPCs were mixed with or without PA-CM or Cpne7 antibody-treated PA-CM, and transplanted into subcutaneous tissue of immunocompromised mice. After twelve weeks of implantation, vascularized pulp-like tissue regenerated inside the root canal spaces in all groups (Fig. 5I-K). In the PA-CM-treated group, odontoblast-like cells exhibited a palisade arrangement on the existing dentinal wall, and their cytoplasmic processes, with lengthened nuclei, extended toward existing dentinal tubules (Fig. 5J, M and P). In the Cpne7 antibody-treated PA-CM group, however, reparative dentin-like mineralized tissues showing entrapped cells was observed between newly formed pulp-like tissue and the existing dentin. BSP was distinctly expressed in the newly formed reparative dentin area of the Cpne7 antibody-treated PA-CM group compared to hDPCs-only and PA-CM-treated group (Fig. 5L-N). However, DSP was clearly detected in the PA-CM group, but only faint staining was observed in the Cpne7 antibody treated PA-CM group (Fig. 5O-Q). Taken together, these in vivo findings indicate that, among preameloblast-derived factors, Cpne7 plays an important functional role in regeneration of the dentin/pulp complex and induces the odontogenic differentiation of mesenchymal cells of dental origin, such as hDPCs.



**Fig. 5. Histological analysis of the regenerated dentin/pulp complex using human dental pulp cells (hDPCs) in vivo.**

(A-H) The hDPCs were mixed with 100 mg HA/TCP particles alone (A, E), or with CM (B, F), CPNE7 antibody-treated CM (C, G), or rCPNE7 (D, H) in a 0.5% fibrin gel and

transplanted subcutaneously into immunocompromised mice for 6 weeks. Samples were stained with hematoxylin-eosin (H-E). (E-H) Boxed areas in A-D are shown at higher magnification in E-H. Scale bars, 200  $\mu$ m. (I-Q) The hDPCs alone (I, L and O) or together with CM (J, M and P) or Cpne7 antibody-treated CM (K, N and Q) in a 0.5% fibrin gel were inserted into the root canal spaces of the human tooth segments for 12 weeks. Regenerated tissues were stained with H-E (I-K) and immunostained with anti-BSP (L-N) and anti-DSP (O-Q). Arrows in J, M and P indicate regenerated odontoblast-like cells with odontoblastic processes. P: regenerated pulp, D: pre-existing dentin wall, RD: newly formed reparative dentin. Scale bars, 100  $\mu$ m.



**Fig. S4. Histomorphometric analysis of mineralized tissue formation in hDPCs in which CPNE7 is overexpressed or inactivated *in vivo*.**

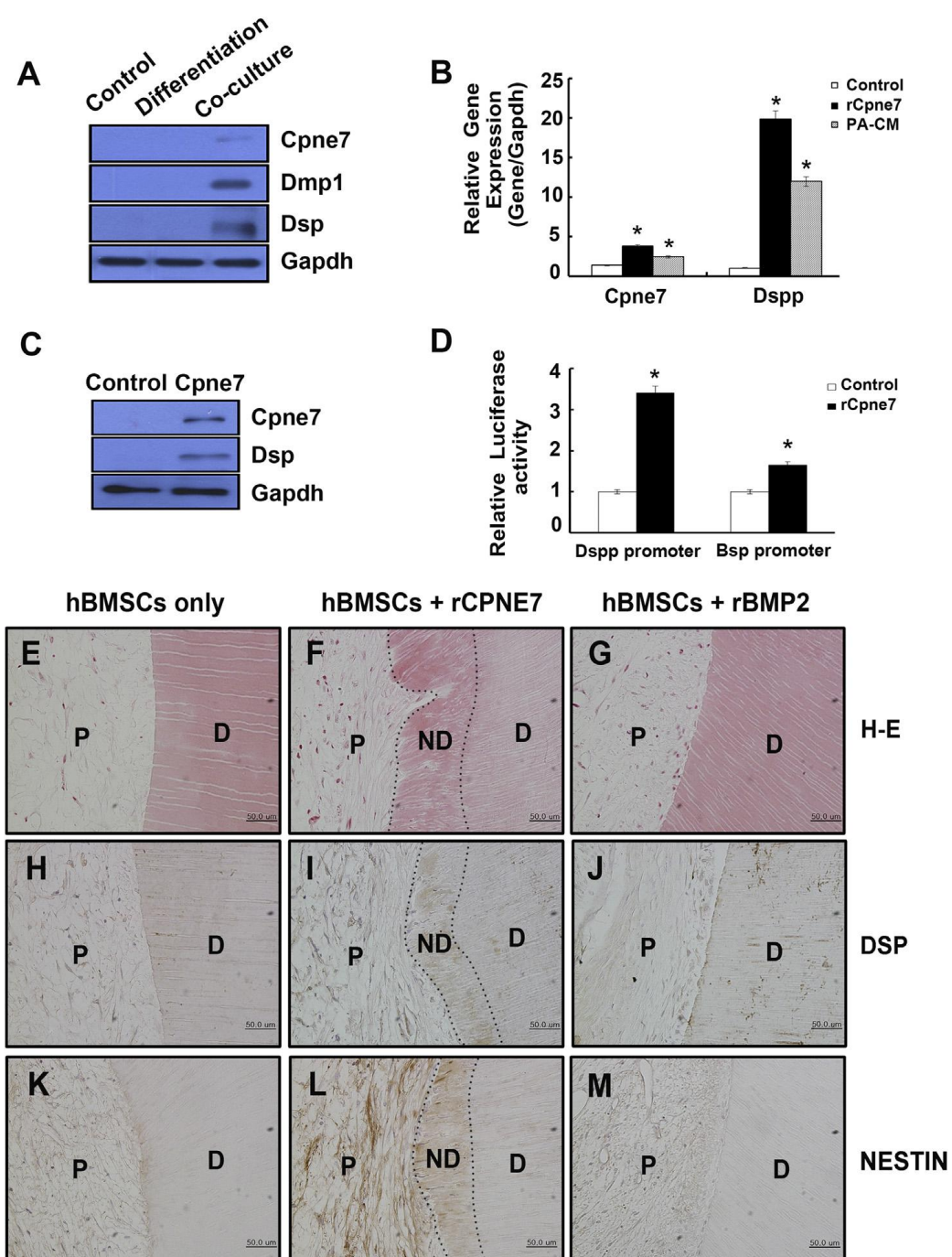
(A-H): The hDPCs were untransfected (A and E) or transfected with CPNE7 overexpression (B and F), CPNE7 siRNA (C and G), or control siRNA (D and H) constructs and transplanted subcutaneously into immunocompromised mice for 12 weeks. Regenerated tissues were stained with H-E. (E-H): Boxed areas in A-D are shown at higher magnification in E-H. Scale bars, 200  $\mu$ m. (I): Total area of generated mineralized tissue in the four groups was analyzed using the LS starter program. All values represent the mean  $\pm$  SD of triplicate experiments. \*  $P < .05$ , \*\*  $P < .01$  compared to control.

## **5. Cpne7 induces differentiation of mesenchymal cells of non-dental origin into odontoblasts in vivo and in vitro**

Our data showed that Cpne7 induced odontoblastic differentiation of hDPCs and promoted dentin-like tissue formation in subcutaneous tissue and empty pulp cavity (Fig. 5). Therefore, we explored the role of Cpne7 in mesenchymal cells of non-dental origin, which indicates that those cells were not induced by dental epithelium previously. We used C3H10T1/2 cells, which are established from mouse embryonic connective tissue and do not differentiate into odontoblast-like cells without induction [32], and human bone marrow mesenchymal stem cells (hBMSCs). First, we examined the effects of ameloblastic ALC co-culture or Cpne7 overexpression in C3H10T1/2 cells. Cpne7 was barely expressed in both control C3H10T1/2 cells and those cells cultured for 5 days in differentiation medium. However, C3H10T1/2 cells co-cultured with ALCs showed Cpne7, Dmp1 and Dsp expression by western blotting (Fig. 6A). Cpne7 and DSPP mRNA expression was also increased after treatment with PA-CM or rCPNE7 (Fig. 6B). In addition, Cpne7 overexpression clearly induced Dsp protein expression in C3H10T1/2 cells (Fig. 6C). Moreover, the Dspp transcriptional activity was more significantly promoted than that of BSP by treatment with rCPNE7 (Fig. 6D). Next, we extended our studies to in vivo experiments using hBMSCs with or without rCPNE7 or rBMP2 proteins in empty root canal spaces for 6 weeks. In the rCPNE7-treated hBMSCs group, these non-dental mesenchymal stem cells differentiated into odontoblast-like cells with odontoblastic cellular processes and dentin-like mineralized tissues, including regeneration of the structure of dentinal tubules on the existing dentinal wall (Fig. 6E-G).

The cellular processes of odontoblast-like cells extended toward the dentinal tubules of newly formed dentin-like tissues, which robustly expressed DSP and NESTIN (Fig. 6H-M). On the other hand, no obvious dentin-like structures or DSP or NESTIN expression were observed in hBMSCs-only and rBMP2-treated hBMSCs groups.

Taken together, these findings suggest that Cpne7 from Preameloblasts might act as a signaling molecule in the odontogenic induction process and, thus, be capable of programming mesenchymal cells of non-dental origin, as well as those of dental origin, into odontoblast-like cells in vivo and in vitro.



**Fig. 6.Effects of Cpne7 on non-dental mesenchymal cells C3H10T1/2 and hBMSCs in vitro and in vivo.**

(A) C3H10T1/2 cells were cultured in induction medium or in co-culture with ALCs. Expression of Cpne7, Dmp1, and Dsp in C3H10T1/2 cells was evaluated by western blotting. (B) Levels of Cpne7 and Dspp mRNA were evaluated by quantitative real-time PCR after 7 days of culture in C3H10T1/2 cells with or without rCPNE7 or PA-CM treatment. All values represent the mean  $\pm$  standard deviation (SD) of triplicate experiments. \*P < .01 compared to control. (C) C3H10T1/2 cells were transfected with Cpne7 overexpression construct. Expression of Cpne7 and Dsp was analyzed by western blotting. (D) Transcriptional activity of Dspp and Bsp promoter was evaluated by luciferase assay after rCPNE7 treatment in C3H10T1/2 cells. All values represent the mean  $\pm$  SD of triplicate experiments. \*P < .05, \*\*P < .01 compared to control. (E-M) hBMSCs alone (E, H and K) or mixed with rCPNE7 (5 mg; F, I and L) or rBMP2 (5 mg; G, J and M) in a 0.5% fibrin gel were inserted into the root canal spaces of the human tooth segments for 12 weeks in vivo. Regenerated tissues were stained by H-E (E-G) and immunostained with anti-DSP (H-J) and anti-Nestin(K-M). Dotted lines in F, I and L indicate the margins of the newly formed dentin, including the structure of dentinal tubules. P: regenerated pulp, D: pre-existing dentin wall, ND: newly formed physiologic dentin. Scale bars, 50  $\mu$ m.



## **6. Cpne7 interacts with nucleolin and regulates odontoblast differentiation via the control of Dspp expression**

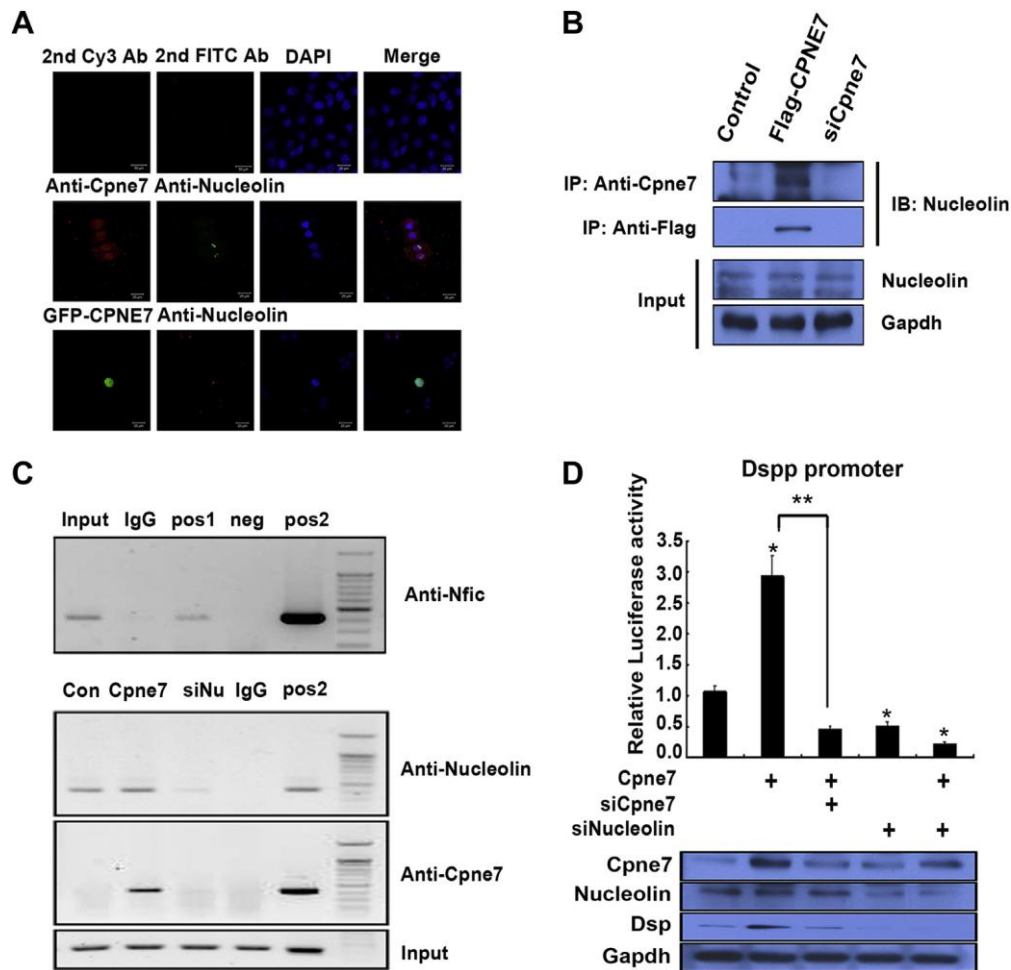
Our data showed that Cpne7 regulated Dspp gene expression in odontoblastic MDPC-23 cells. However, there is no DNA-binding motif in the structure of copine family members [33, 34]. Therefore, we further investigated other potential effectors of Cpne7-mediated Dspp expression because copine family proteins contain two distinctive domains, a phospholipid-binding domain and a protein interaction domain [9]. Nucleolin is a multifunctional protein that is involved in many cellular activities, including cell proliferation, embryogenesis, and cell death [35-38]. Nucleolin plays a functional role in tooth development [39] and interacts with Cpne3 [12].

To provide further evidence for an interaction between Cpne7 and Nucleolin, we performed immunocytochemical and coimmunoprecipitation (co-IP) analyses of MDPC-23 cells. Notably, both endogenous and exogenous Cpne7 proteins showed strong colocalization with Nucleolin protein in nuclei of odontoblasts (Fig. 7A) and interacted with Nucleolin (Fig. 7B). Next, we investigated whether the Cpne7-Nucleolin complex binds specifically to the Dspp promoter. A positive control was designed using the well-characterized nuclear factor I-C antibody on DSPP promoter [40]. Chromatin immunoprecipitation (ChIP) assay revealed that Nucleolin bound to the Dspp promoter, whereas negative control (pre-immune serum) or Nucleolin siRNA did not precipitate the Dspp promoter fragment (Fig. 7C, Middle panel). Also, Cpne7 remained bound to the Dspp promoter (Fig. 7C, Lower panel). To confirm that Nucleolin is sufficient to interact with the AP-1 site of the Dspp promoter, we used a DNA affinity protein-binding assay

(DAPA) with biotin-labeled AP-1 site. Nucleolin was associated with the wild-type AP-1 site, but not with the mutated sequence (Supporting Information Fig. S5).

Finally, to further investigate the effect of Nucleolin on Cpne7-mediated Dspp transcription, we transfected MDPC-23 cells with Nucleolin siRNA constructs and conducted luciferase reporter assays using a Dspp-responsive reporter. In these cells, Nucleolin level was reduced by Nucleolin siRNA, without alteration of Cpne7 level (Fig. 7D, Lower panel). Cpne7 stimulated Dspp promoter activity and this activation was abolished with the knockdown of Nucleolin (Fig. 7D, Upper panel). These data indicated that Nucleolin is critical for Cpne7-dependent Dspp transcription.

Taken together, the results suggest that Cpne7 as a coregulator is physically associated with Nucleolin protein, and the Cpne7-Nucleolin complex modulates Dspp gene transcription.

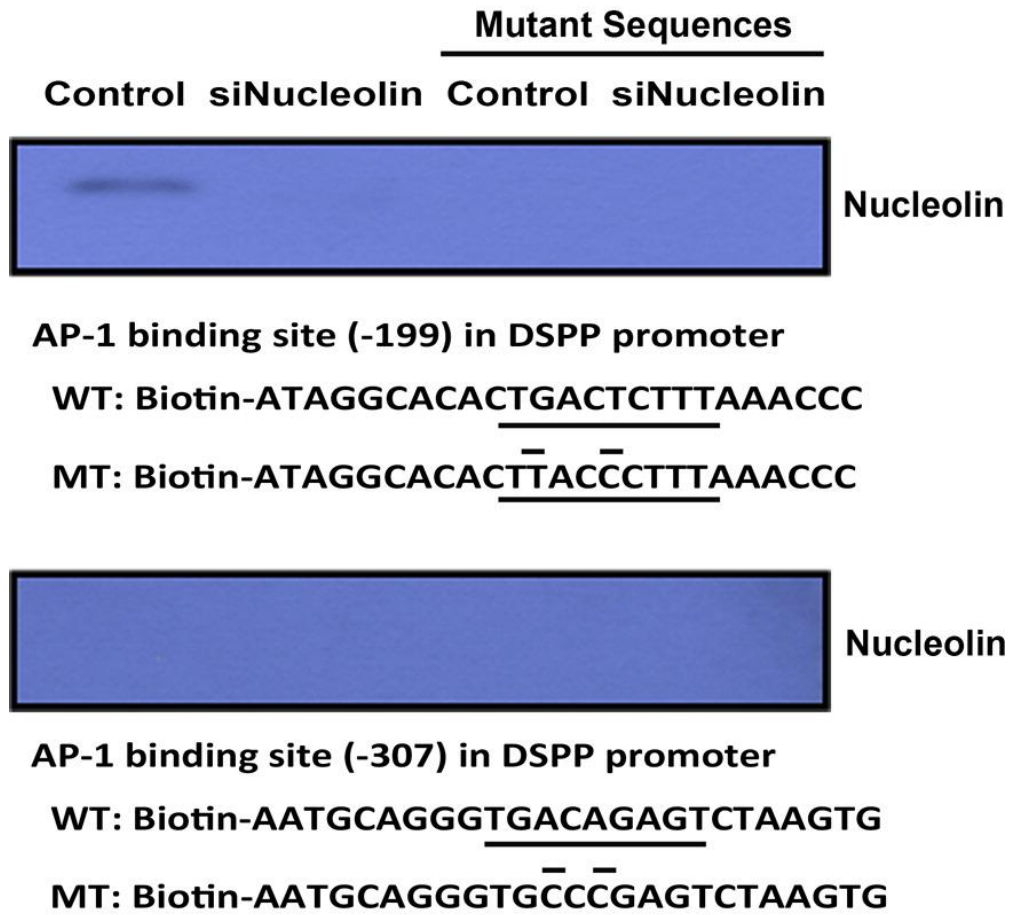


**Fig. 7. Dspp is regulated by a complex of Cpne7 and Nucleolin.**

(A) Co-localization of Cpne7 and Nucleolin was detected by fluorescence microscopy in MDPC-23 cells (Scale bars: 20 $\mu$ m). (B) MDPC-23 cells were transfected with Flag-tagged CPNE7 expression or Cpne7 siRNA construct. Immunoprecipitated (IP) Cpne7 and the whole cell lysates (Input) were analyzed by western blotting (IB) with anti-Nucleolin antibody. (C) Cross-linked chromatin was prepared and immunoprecipitated with pre-immune serum (IgG) or Nfic-specific antibody in MDPC-23 cells (Upper panel).

ChIP assays were performed using anti-Nucleolin, anti-Cpne7, or IgG antibodies. Chromatin samples were subjected to PCR analysis using primer pairs spanning the AP-1 site on the Dspp promoter. Input: the PCR product of chromatin obtained before immunoprecipitation, IgG: pre-immune serum, pos1: ChIP positive (anti-Nfic), neg: PCR negative, pos2: PCR positive, con: control, siNu: Nucleolin siRNA. (D) MDPC-23 cells were cotransfected with the indicated vectors. The transcriptional activity of the Dspp promoter was evaluated by luciferase assay. Expression levels of Cpne7, Nucleolin, and Dsp in MDPC-23 cells were evaluated by western blotting. All values represent the mean  $\pm$  SD of triplicate experiments. \*P < .05, \*\*P < .01 compared to control.

## MDPC-23



**Fig. S5. Nucleolin binds to the *Dspp* promoter.**

MDPC-23 cells were transfected with Nucleolin siRNA, and nuclear extracts were prepared and mixed with biotinylated *Dspp* promoter probes containing wild-type (WT) and mutant (MT) AP-1 sites. Nucleolin in the complex was detected by western blotting.

## V. DISCUSSION

Morphogenesis and differentiation of organs are regulated by short-range diffusible signals, commonly referred to as epithelial-mesenchymal interactions [41]. At the bell stage of tooth development, undifferentiated ectomesenchymal cells become enlarged and are going to differentiate into odontoblasts after induction by dental epithelial cells, which involve paracrine interactions [42]. In this paper, we focus on molecular mechanisms underlying dentinogenesis in the developing crown via epithelial-mesenchymal interactions. First, we observed expression and secretion of Cpne7 only in early stages of ameloblast differentiation, but in early to middle stages of odontoblast differentiation. Second, we demonstrated translocation of Cpne7 from preameloblasts to differentiating odontoblasts. Results of our histological analysis showed that Cpne7 was expressed only in epithelial cells, including inner enamel epithelium and stratum intermedium at E19. At P7 and P10, Cpne7 existed in odontoblasts, pre-dentin, and dentinal tubules, although Cpne7 was no longer expressed in epithelial tissue, continuously. Further, Cpne7 protein was translocated from ameloblasts into odontoblasts in the co-culture system. Therefore, we first identified Cpne7 as a new mediator protein in the molecular mechanism of epithelial-mesenchymal interactions during dentinogenesis in the developing crown. Moreover, we demonstrated that the stage of the shift in Cpne7 localization from the epithelium to the underlying mesenchyme corresponds temporospatially to that of the initiation of odontoblast differentiation.

Next, we explored the function of Cpne7 and how it controls odontoblast differentiation during tooth development. Based on previous reports and our data, Cpne7 mRNA and

protein increased during odontoblast differentiation, and stimulation of Cpne7 promotes expression of odontoblast-related genes, including Dspp, Osteocalcin, and Alp [7]. Specifically, we observed that the expression of Dspp (mRNA, protein, and transcriptional activity) was upregulated by Cpne7 overexpression or rCPNE7 treatment, and rCPNE7 promoted mineralized nodule formation in vitro. In addition, inactivation of Cpne7 in PA-CM by Cpne7-specific antibody prevented induction of dentin-like mineralized tissue formation in transplantation experiments in vivo. Indeed, Cpne7 had a significant effect on formation of new dentin-like structures in mesenchymal cells of both dental and non-dental origin in vivo.

These findings indicate that Cpne7 is indispensable and also plays important roles in regulating odontoblast differentiation and mineralization.

In the present study, we have used odontoblastic cell line, MDPC-23, to evaluate the expression of Cpne7 in odontoblasts. As a result, endogenous Cpne7 was expressed in MDPC-23 cells from the beginning of the culture without PA-CM induction. It is well known that MDPC-23 cells can differentiate into odontoblasts without dental epithelial induction because the cells were already induced by underlying inner enamel epithelium previously, so that they could express Cpne7. Endogenous Cpne7 expression was enhanced by co-culture with ALCs or rCPNE7 treatment. In addition, Cpne7 was localized in both the cytoplasm and the nucleus of differentiating odontoblasts in early to middle stages, but secreted into extracellular matrix in late stage. These results imply that exogenous Cpne7 enters differentiating odontoblasts, induces endogenous Cpne7 expression, and triggers the autocrine/paracrine secretion of Cpne7 from differentiating

odontoblasts similar to that of the Bmp [43]. Once triggered by dental epithelial cell-derived Cpne7, differentiating odontoblasts can secrete Cpne7 by themselves, which acts in autocrine/paracrine manner.

Our in vivo transplantation studies establish that Cpne7 induces dentin-like mineralized tissue formation in hDPCs with HA/TCP. The formation of a dentin/pulp-like complex was more extensive in rCPNE7-treated or Cpne7-overexpressing hDPCs groups than in the hDPCs-only group. The upregulation of DSP in the rCPNE7 treatment group indicated that Cpne7 promoted differentiation of hDPCs into cells with more odontogenic characteristics. Moreover, the Cpne7-inactivated CM group showed formation of reparative dentin-like structure containing cells entrapped in the mineralized matrix and expression of BSP, which leads to failure of complete physiological dentin regeneration. Therefore, Cpne7 is critical for the formation and regeneration of a dentin/pulp complex in vivo.

Nucleolin is a multifunctional protein and directly or indirectly plays a role in the regulation of cell proliferation, differentiation, and apoptosis. Nucleolin is composed of three domain structures, an acidic histone-like N-terminus, an RNA-binding domain, and an arginine-and-glycine-rich domain. Nucleolin binds various kinds of proteins, such as midkine (MK) in proliferation [44], STAT1 in monocyte differentiation[45], and Fas in apoptosis [46]. Indeed, Nucleolin recognizes sequence-specific DNA-binding sites, including the AP-1 (TGAC/gTCA) and SP-1 (AGCCCA and AGCCCCT) sites, and regulates cPLA2a gene transcription [47, 48]. Computer analysis demonstrates that the promoter region of the Dspp gene contains various potential binding sites of transcription



factors AP-1, AP-2, Runx2, C/EBP, and NF-1 [49]. A previous study showed that Nucleolin is expressed in the inner enamel epithelium, the cusp areas of the dental papilla, and cervical loop during the crown formation stage [39]. In addition, CPNE3 binds Nucleolin through the vWA domain, which is involved in protein-protein interaction in SKBr3 cells [12]. Similarly, our present study showed that Cpne7 and Nucleolin were co-localized and interacted in MDPC-23 cells. In addition, Nucleolin and Cpne7 bound the Dspp promoter and regulated Dspp gene expression. Based on previous reports and our data, we suggest that the Cpne7-Nucleolin complex might regulate transcriptional activation of the Dspp promoter and be responsible for Cpne7-mediated Dspp expression during odontoblast differentiation and mineralization.

## **CHAPTER III.**

### **Zinc Balance is Critical for NFI-C Mediated Regulation of Odontoblast Differentiation**

**\* This Chapter has been largely reproduced from an article published by OH HJ.  
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## **I. ABSTRACT**

Zinc is trace element essential for diverse metabolic and cellular signaling pathways for the growth, development, and maintenance. Zinc deficiency is involved in bone malformations and oral disease. Mice deficient in zinc transporter Zip13 show connective tissue and skeletal disorders, abnormal incisor teeth, and reduced root dentin formation in the molar teeth and share a morphologically similar phenotype to nuclear factor I-C (NFI-C)-deficient mice. However, the precise function of zinc in NFI-C signaling-mediated odontoblast differentiation and dentin formation remains unclear. Here, we show that zinc stimulated the expression of metal transcription factor-1, but decreased NFI-C expression in odontoblastic MDPC-23 cells. Zinc also enhanced the phosphorylation of Smad2/3 (p-Smad2/3) and increased the binding efficiency of NFI-C and p-Smad2/3 in the cytoplasm. In contrast, zinc deficiency resulted in the accumulation of NFI-C into nucleus. Consequently, NFI-C had the biologic properties of a transcription factor, including DNA binding affinity for metallothionein-1 and the dentin sialophosphoprotein (DSPP) promoter, and transcriptional activation of the DSPP gene. Furthermore, zinc deficiency condition promoted DSPP expression in odontoblasts and dentin mineralization, while zinc sufficiency condition decreased DSPP expression and slightly delayed dentin mineralization. These data suggest that zinc equilibrium is required for odontoblast differentiation and dentin formation during dentinogenesis through the nuclear accumulation and modulation of NFI-C.

## II. INTRODUCTION

The trace element zinc is essential for growth, development, and health maintenance, and is involved in cellular metabolism, replication, growth, and tissue repair [17]. Zinc modulates transcription and other biological functions through more than 300 zinc containing enzymes and 2,000 transcription factors that contain a DNA-binding Zn-finger motif, a Ring-finger, and an LIM domain [18]. Consequently, intracellular zinc concentrations are extremely important for the maintenance of regulatory systems. Moreover, zinc deficiency results in malformation and retardation of bone growth [19] as well as the impairment of immune responses and brain functions [20, 21]. Conversely, excess zinc can induce apoptosis and neuronal death [22]. Therefore, it is important not only the element of zinc but also the equilibrium in intracellular zinc concentrations for mediating proper intracellular signaling events. Zinc homeostasis is tightly controlled by various proteins, which include zinc transporters, zinc binding molecules, such as metallothioneins (MTs), and zinc sensing molecules, such as metal transcription factor-1 (MTF-1). MTF-1 is a six-zinc finger domain-containing transcription factor that induces the expression of MTs in response to heavy metal ions, such as zinc. MTs play important roles in zinc homeostasis [50-52]. The nuclear factor I (NFI) family of transcription factors consists of four members (Nfia, Nfib, Nfic, and Nfix) in mammals. NFI proteins contain an N-terminal DNA-binding/dimerization domain and a C-terminal NFI transactivation and repression domain [13]. Interestingly, Nfic-deficient mice exhibit thin and brittle mature mandibular incisors, dimorphic maxillary incisors, and the absence of a molar root. Thus, NFI-C is important for odontoblast differentiation, which is essential

for the production of the thick dentin layer that forms the bulk of the tooth [16]. The zinc transporter Slc39a13/Zip13 influences intracellular zinc levels by transporting zinc from the Golgi to the cytosol. Mice that are deficient for the zinc transporter Zip13 show connective tissue and skeletal disorders, abnormal incisor teeth, and reduced root dentin formation in the molar teeth [23]. Interestingly, these mice share a morphologically similar phenotype to Nfic-deficient mice [15]. However, the precise mechanism by which zinc regulates the NFI-C gene for odontoblast differentiation and dentin formation remains unknown. Transforming growth factor beta (TGF- $\beta$ ) is a multifunctional cytokine that regulates cellular processes, such as mediating odontoblast differentiation and dentin formation [53]. During TGF- $\beta$  signaling, the activated TGF $\beta$ -R1 phosphorylates Smad2 and Smad3, which form a complex with a common partner, Smad4, which then translocates into the nucleus to control the transcription of target genes [54]. There are many studies showing that zinc is associated with osteogenic regulatory genes of bone formation and clinical dental studies. Indeed, zinc can promote osteoblastic bone formation and inhibit osteoclastic bone resorption [55, 56]. In addition, zinc insufficiency has been related to defective collagen synthesis and delayed skeletal maturation [57, 58]. Furthermore, zinc deficiency has been linked to oral disease, such as human taste impairments, parakeratosis in the black plaque buccal epithelium [59], and susceptibility to dental caries [60, 61]. However, the role of zinc in the odontoblast differentiation and dentin mineralization has not yet been clearly defined.

The present study was conducted to assess how NFI-C signaling and zinc concentration changes affect odontoblast differentiation and mineralization in a mouse odontoblast cell

line (MDPC-23). We also determined whether zinc equilibrium is required for dentin formation through the nuclear accumulation and modulation of NFI-C.

### **III. MATERIALS AND METHODS**

#### **1. Cell culture**

MDPC-23 cells were provided by Dr. J.E. Nordin (University of Michigan, MI). 293T (human embryonic kidney) and MC3T3-E1 (mouse osteoblast-like) cells were obtained from ATCC (Rockville, MD, USA). MDPC-23 and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM), and MC3T3-E1 cells were cultured in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum and antibiotic-antimycotic (Invitrogen, Carlsbad, CA) in a 5% CO<sub>2</sub> atmosphere at 37°C. To induce cell differentiation and mineralized nodule formation, confluent MDPC-23 cells were treated with 50 mg/ml ascorbic acid and 10mM  $\beta$ -glycerol phosphate for up to 2 weeks.

#### **2. Plasmids**

Flag-tagged Smad3 cDNA was placed on a pcDNA3 vector (Invitrogen). HA-tagged NFI-C was constructed with a pcDNA3 vector (Invitrogen) from Dr. R. M. Gronostajski (State university of New York at Buffalo, Buffalo, NY). The green fluorescent protein (GFP)-tagged NFI-C gene was placed into pEGFP-C3 (BD Biosciences, San Jose, CA). The dentin sialophosphoprotein (DSPP) promoter (pGL3LUC-791 to p54) plasmid was a kind gift from Dr. W.X. He (Qin Du Stomatological, Xian, China). A 1.5-kb genomic fragment was amplified from mouse genomic DNA to obtain the NFI-C promoter (-1,543 to +88). One microliter of the mouse genomic DNA was subjected to PCR using the following cycling conditions: 94°C for 1 min; 60°C for 1 min; and 72°C for 1 min for a total of 35

cycles. The forward and reverse primers were as follows: 5'-CTC GAG GGA CTG TAA CTG CTG AGC TGT-3' and 5'-AAG CTTCAG AGC GGG GAG GAA TAC AT-3'. The amplified 1.5-kb fragment was subcloned into PCR1 2.1 T vector and ligated into the XhoI and Hind III sites of pGL3 luciferase (LUC) basic expression vector (Promega, Madison, WI). The construct was confirmed by sequencing and was designated pGL3-mNFI-C.

### **3. Colorimetric MTT assay for cell proliferation**

MDPC-23 cells were seeded at a density of  $5 \times 10^3$  cells per well in 48-well culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing various concentrations of ZnCl<sub>2</sub> (Sigma-Aldrich, St. Louis, MO) and cell membrane-permeable zinc chelator, N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, Sigma-Aldrich) for 24 h. The mitochondria activity of proliferating cells was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The plates were measured at 540 nm using a plate reader. Triplicate samples were analyzed from three independent experiments.

### **4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted using TRIzol Reagent (Invitrogen), and 3 µg of RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (New England Biolabs, Beverly, MA). One microliter of the RT product was amplified by PCR using the following primer pairs: MT-1 (399 bp), forward, 5'-



gtggtccggaacctcaaagt-30 and reverse, 50-ccggagcaggatagcaaagt-30; MTF-1 (289 bp), forward, 50- 50-cctgcagtcacgcttagtca-30 and reverse, 50-tggggaagaacatccctttc-30; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 452 bp), forward, 50-accacagtccatgccatcac-30 and reverse, 50-tccaccaccctgtgctgt-30. The following PCR conditions were used: 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 1 min for a total of 30 cycles. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

## **5. Preparation of cytoplasmic and nuclear protein extracts**

Zinc or TPEN was administered to MDPC-23 cells dose-dependently for 4 h. At the end of each experiment, the cells were collected by centrifugation at 3,000 rpm for 5 min at 4 °C. Cell lysis was performed in ice-cold hypotonic lysis buffer (10mM HEPES [pH 7.9], 10mM KCl, 0.1% NP-40) supplemented with protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) for 15 min. Nuclear and cytoplasmic fractions were separated by centrifugation at 3,000 rpm for 5 min at 4 °C. The resulting supernatant (the cytoplasmic fraction) was stored at 4 °C until further analysis. The membrane pellet was resuspended in ice-cold hypertonic lysis buffer (10mM HEPES [pH 7.9], 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol) supplemented with protease inhibitors and incubated for 15 min at 4 °C. The soluble fraction was isolated by centrifugation at 3,000 rpm for 5 min at 4 °C. The resulting supernatant (the nuclear fraction) was stored at 4 °C until further analysis.

## 6. Western blot analysis

To prepare whole cell extracts, the cell were washed three times with PBS, scraped into 1.5-ml tubes, and pelleted by centrifugation at 12,000 rpm for 5 min at 4°C. After removal of the supernatant, the pellet was resuspended in lysis buffer (50mM Tris-Cl [pH 7.4], 150mMNaCl, 1% NP-40, 2mM EDTA [pH 7.4]) and incubated for 15 min on ice. Cell debris was removed by centrifugation at 12,000 rpm for 15 min at 4°C. The proteins were resuspended in lysis buffer as described above. Thirty microgram of samples were separated on denaturing 10–12% Tris-HCl polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% nonfat dry milk in phosphate buffered saline containing 0.1% Tween 20 (PBS-T), washed with the PBS-T, and incubated overnight with primary antibody diluted in PBS-T buffer (1:1,000) at 48 °C. Antiserum against NFI-C was produced by immunization in rabbit with the synthetic peptides NH<sub>2</sub>-RPTRPLQTVPLWD-COOH (amino acid residues 427-439 of NFI-C) and NH<sub>2</sub>-GNKSIITKESGKLSGS-COOH (amino acid residues 372–387 of DSP) [15]. The mouse monoclonal anti-HA (E10176EF) and anti-Flag (F-3165) antibodies were purchased from Sigma–Aldrich. The antibodies against p-Smad2/3 (sc-11769), MTF-1 (sc-48775), Smad 1/5/8 (sc-6031) p-Smad 1 (sc-101800), Lamin B (sc-6216), CDK2 (sc-6248), osteocalcin (sc-30044), Runx2 (sc-10758), and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-Smad3 antibody (9523) was purchased from Cell Signaling Technology (Beverly, MA). After washing, the membranes were incubated with antimouse (sc-2031), -rabbit (sc-2004), or -goat (sc-2768) IgG secondary antibodies conjugated to horseradish peroxidase (Santa

Cruz Biotechnology) for 1 h. The labeled protein bands were detected using an enhanced chemiluminescence system (Dogen, Cambridge, MA), and the bands were measured by densitometric analysis of the autoradiograph films.

## **7. Fluorescence microscopy**

MDPC-23 cells in Laboratory-Tek chambered cover glasses (Nunc, Wiesbaden, Germany) were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized for 4 min in PBS containing 0.5% Triton X-100. After washing, the cells were incubated with anti-NFI-C antibody (1:200 dilution) in blocking buffer (PBS and 1% bovine serum albumin) for 1 h followed by the addition of a fluorescein isothiocyanate conjugated anti-rabbit IgG antibody (1:200 dilution; Amersham Pharmacia Biotech, Orsay, France). After washing, the cells were visualized using a fluorescence microscope (AX70; Olympus Optical Co., Tokyo, Japan). DAPI (40, 6-diamidino-2-phenylindole) was used to stain chromosomal DNA in the nucleus.

## **8. Transient transfection and luciferase assay**

293T cells were seeded in 12-well culture plates at a density of  $1.5 \times 10^5$  cells per well. Cells were transiently transfected with the reporter constructs described above. Depending on the experimental conditions, pGL3-NFI-C and pGL3-DSPP was transfected into the cell, which were treated with zinc or TPEN (dose-dependent) 2 days later. Following the addition of 50  $\mu$ l Luciferin to 50  $\mu$ l of the cell lysate, the luciferase activity was determined using an Analytical Luminescence Luminometer according to the

manufacturer's instructions (Promega).

## **9. Co-immunoprecipitation (CO-IP) assay**

After transfection with the indicated plasmid DNA using Metafectene pro reagent (Biontex, Munich, Germany), 293T cells were washed in PBS, and the cell lysates were prepared by adding 1ml of Co-IP buffer (50mM Tris-Cl, pH 7.5, 150mM NaCl, 0.1% NonidetP-40, 5mM ethylenediaminetetraacetic acid [EDTA]) supplemented with protease inhibitors (Roche Molecular Biochemicals). The lysates were incubated at 48C for 2 h with a 1:200 dilution of mouse monoclonal anti-HA antibodies. Thirty microliter of proteinA/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology) was added and incubated at 4°C for 1 h with rotation. The immune complexes were then released from the beads by boiling in sample buffer for 5 min. Following electrophoresis on 10% SDS-polyacrylamide gels, the immunoprecipitates were analyzed by Western blotting with either anti-HA or anti-Flag antibodies.

## **10. Chromatin immunoprecipitation (ChIP) assay**

MDPC-23 cells were treated with zinc or TPEN. The cells were treated with the cross-linking reagent formaldehyde (1% final concentration) for 10 min at 37 °C, rinsed twice with cold PBS, and swollen on ice in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl [pH8.1]) for 10 min. The nuclei were collected and sonicated on ice. The supernatants were obtained by centrifugation for 10 min and were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl [pH

8.1], and 167mM NaCl).The fragmented chromatin mixture was incubated with anti-MTF-1(1ml) and anti-NFI-C antibodies (30μl) on a rotator at 4 °C for 4 h. Thirty microliter of protein A/G PLUS-agarose (Santa Cruz Biotechnology) was added and incubated at 4 °C for 1 h with rotation to collect the antibody/chromatin complex. Cross-linked, precipitated chromatin complexes were recovered and reversed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid,NY). The final DNA pellets were recovered and analyzed by PCR using primers that encompass the MT-1 promoter region (183 bp); mouse MT-1 promoter 238 region: forward, 50-cactatagggacatgatgttc-30 and mouse MT-1 promoter 55 region: reverse, 50-cagcaggcggttgctcca-30, and the DSPP promoter region (390 bp),mouse DSPP-400 region: forward 50-gggtcttaaatagccagtcg-30 and mouse DSPP-10 region: reverse, 50-ctgagagtggcacactgt-30. The following PCR conditions were used: 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 1 min for a total of 35 cycles. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

## **11. Alizarin red S staining**

The cells were fixed with 70% ethanol for 20 min and stained with 1% alizarin red S (Sigma–Aldrich) in 0.1% NH<sub>4</sub>OH at pH 4.2–4.4. The mineralization assays were performed by staining MDPC-23 cells treated with either zinc or TPEN with alizarin red S solution. The cells were evaluated at 0, 4, 7, 10, and 14 days. To quantify the intensity of mineralization, we measured density of stained nodules by colorimetric spectrophotometry. The stained cells were collected by centrifugation at 13,000 rpm for

10 min at 4 °C. Cell lysis was solubilized with 0.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature (vortexing or pipetting). Solubilized stain (0.1 ml) was transferred to wells of a 96-well plate, and absorbance was measured at 405 nm.

## **12. Statistical analyses**

Statistical analyses were carried out using a Student's t-test. All statistical analyses were performed using SPSS software ver. 19.0.

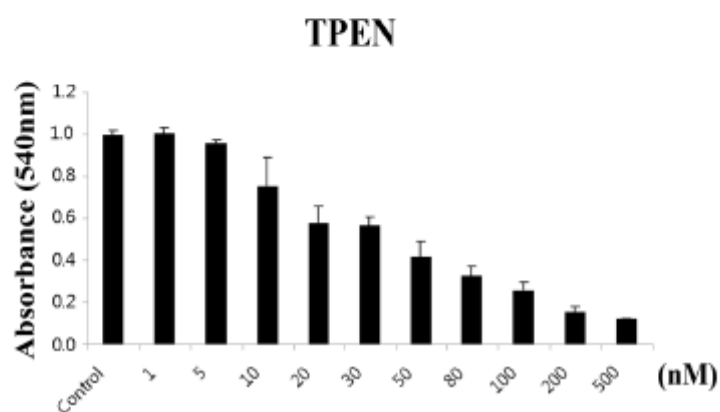
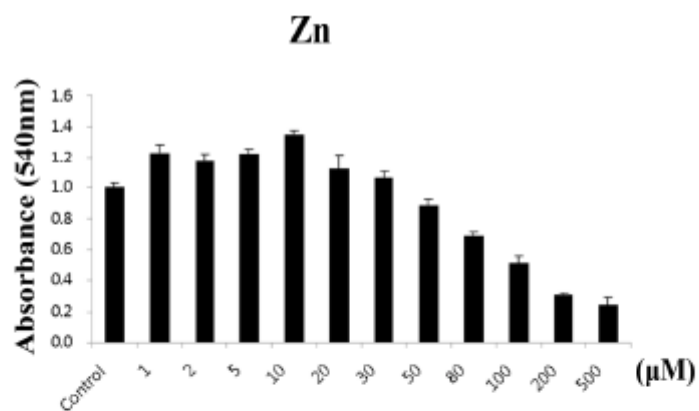
## IV. RESULTS

### 1. Effect of zinc on NFI-C gene expression in odontoblasts

Physiological concentration of zinc is 1.5–100  $\mu$ M in Hep-2 cells[62]. To select suitable doses of physiological concentration, we analyzed the cell viability by MTT assay. We treated various concentrations of zinc and zinc chelator, TPEN, in mouse odontoblast cells (MDPC-23). In the present study, odontoblast cell viability was increased in additive zinc concentration (10–30  $\mu$ M) and decreased in a dose dependent manner of high zinc level and TPEN (supplemental Fig. S1). Therefore, we had chosen 10, 30, 50, or 80  $\mu$ M concentration of zinc and 1, 5, or 10nM concentration of TPEN for experiments. Cellular zinc homeostasis and zinc cellular signaling processes are controlled by MTs [50]. Also, intracellular zinc concentrations and MT gene expression are dependent on the supplement of zinc or zinc chelator in THP-1 cells [63]. When compared with levels in control cells, MT-1 mRNA increased in MDPC-23 cells following the addition of zinc to the culture medium. However, MT-1 mRNA levels decreased after the addition of TPEN (Fig. 1A). Similar to previous reports, these data showed that changes in MT-1 mRNA expression are linked to zinc or TPEN mediated changes in intracellular zinc concentrations in MDPC-23cells. We further investigated whether zinc is involved in the regulation of the MTF-1, NFI-C, Smad 2/3, and Smad 1/5/8 genes in odontoblasts. Zinc treatment enhanced the expression of MTF-1mRNA (Fig. 1A) and protein (Fig. 1B), but decreased NFI-C protein expression. However, TPEN treatment hardly influenced NFI-C expression. In addition, zinc also enhanced phosphorylation of theSmad2/3 protein (p-Smad2/3) and Smad 1 protein (p-Smad 1) (Fig. 1B). To confirm the zinc-mediated

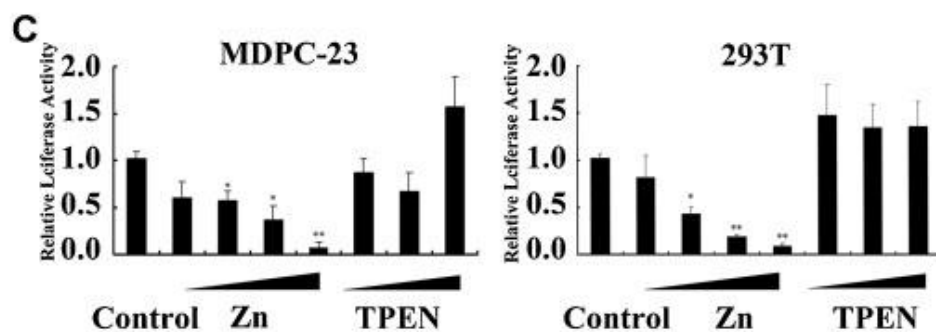
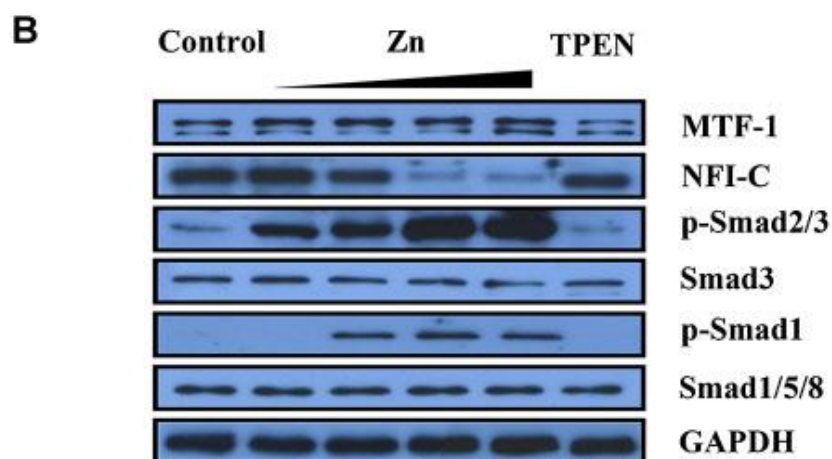
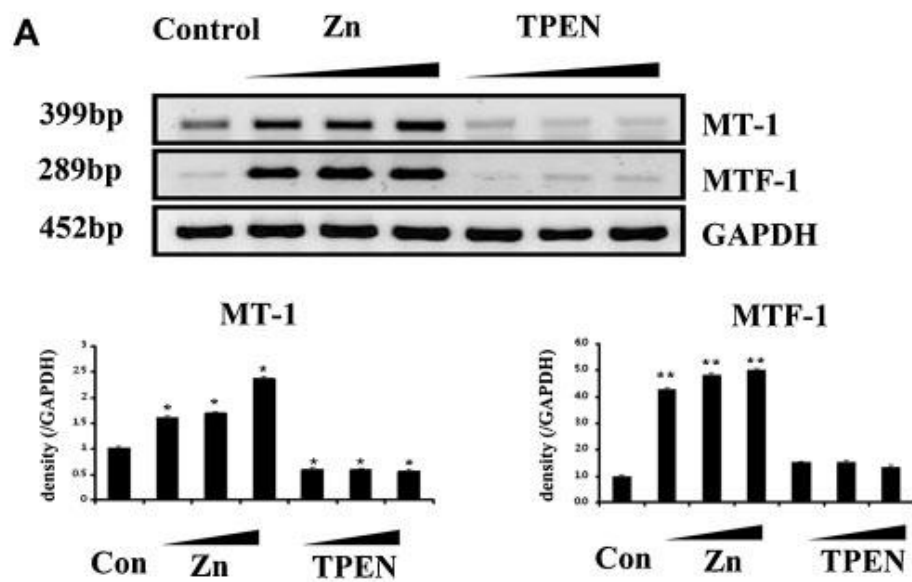
expression change in NFI-C, we performed an NFI-C promoter assay. We discovered that NFI-C promoter activity was decreased by zinc in a concentration dependent manner. However, TPEN barely influenced NFI-C promoter activity in both MDPC-23 and 293T cells (Fig. 1C). These results support the hypothesis that intracellular zinc concentrations change NFI-C expression in odontoblasts.





**Fig.S1. Evaluation of cell viability using MTT assay.**

MDPC-23 cells were untreated or treated with either increasing concentration of zinc or TPEN and analyzed for viable cells by MTT assay as described in Materials and Methods. The data are presented as the mean  $\pm$  SD of triplicate experiments.



**Fig. 1. MDPC-23 cellular expression of MT-1, MTF-1, NFI-C, Smad 2/3, and Smad1/5/8 mRNA and protein in the presence or absence of zinc.**

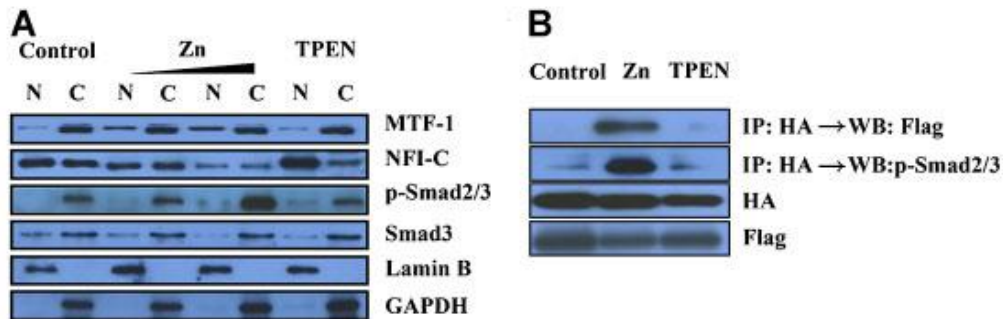
(A) MDPC-23 cells were treated with 10, 50, or 80 mM of zinc or 1, 5, or 10 nM of TPEN. MT-1 and MTF-1 expression were monitored by RT-PCR. Densitometry analysis of the MT-1 and MTF-1 gel images (lower panel). Con; control. Zinc induced MT-1 and MTF-1 mRNA expression. The data are presented as the mean $\pm$ SD of triplicate experiments. Asterisks denote values significantly different from the control (\*P <0.05; \*\*P <0.01). (B) MDPC-23 cells were treated with 10, 30, 50, or 80 mM of zinc or 10 nM of TPEN. The cell lysates were analyzed by Western blot using antibodies against MTF-1, NFI-C, p-Smad 2/3, Smad3, p-Smad 1, Smad 1/5/8, and GAPDH. Zinc increased MTF-1 protein levels and induced the phosphorylation of Smad2/3 and Smad 1, but decreased NFI-C expression. (C) MDPC-23 and 293T cells were transfected with the NFI-C reporter gene. Cells were treated with increasing concentrations of zinc (10, 30, 50, and 80 mM) or TPEN (1, 5, and 10 nM) for 4 h, and the cell lysates were subjected to luciferase assays. The activity of the NFI-C promoter was decreased after zinc administration. Promoter activity was determined as luciferase light units/protein and expressed as fold activation compared to control. The data are presented as the mean $\pm$ SD of triplicate experiments (\*P <0.05; \*\*P <0.01).

## **2. Effect of zinc on the subcellular localization of NFI-C**

A number of stress-responsive transcription factors are primarily localized to the cytoplasm, but they can translocate to the nucleus when required. Like these, MTF-1 has a dual nuclear and cytoplasmic localization. MTF-1 is normally found in the cytoplasm; however, it translocates into the nucleus and induces the expression of MTs in response to zinc in the human kidney [64]. NFI-C2 is also translocated into the nucleus upon prolactin stimulation in mammary glands cells [65]. To examine the effect of zinc on the intracellular localization of MTF-1, NFI-C, and p-Smad2/3, we isolated nuclear and cytoplasmic protein fractions and investigated their cellular localization by Western blot analysis. Zinc promoted the rapid nuclear translocation of MTF-1 in MDPC-23 cells (Fig. 2A). In control cells, endogenous NFI-C was located in the cytoplasm and the nucleus. In cells that received 50 mM of zinc for 4 h, we did not observe a change in localization pattern for NFI-C compared to the control. However, in zinc deficiency, endogenous NFI-C was localized and accumulated in the nucleus (Fig. 2A and supplemental Fig. S2). Similar with endogenous NFI-C, exogenous GFP-tagged NFI-C also accumulated in the nucleus of MDPC-23 cells (supplemental Fig. S2). Moreover, Smad2/3, which is phosphorylated by zinc, was observed in cytoplasm in a dose-dependent manner. However, the activity and nuclear translocation of p-Smad2/3 were not altered by TPEN (Fig. 2A).

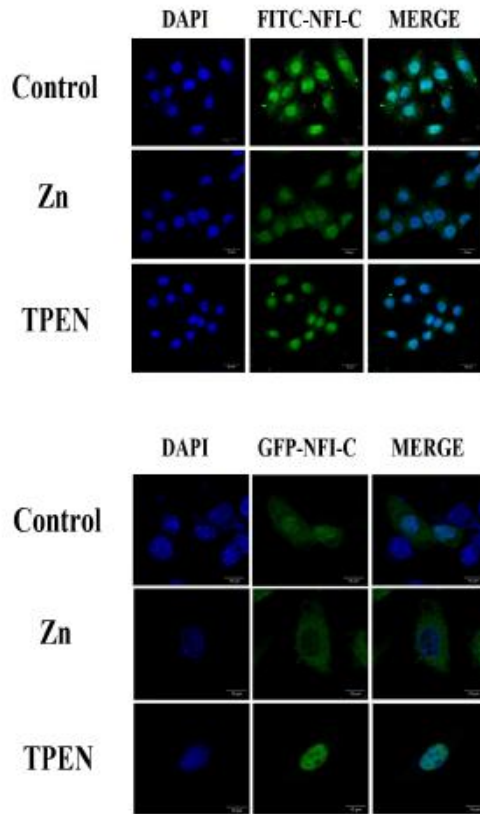
TGF- $\beta$  regulates proliferation, cellular differentiation, and other functions in most cells [66]. During dentinogenesis, Smads and NFI-C signaling work via negative feedback. Indeed, TGF- $\beta$  receptor 1 and Smad3 are over-expressed in *Nfic*-deficient mice [15].

TGF- $\beta$  induced the interaction between p-Smad3 and NFI-C, leading to the degradation of NFI-C (unpublished data). We examine that zinc has the capacity to induce the interaction between p-Smad2/3 and NFI-C. 293T cells were transfected with an HA-tagged NFI-C expression construct and a Flag-tagged Smad3 expression construct for IP assay. The IP assay with an anti-HA antibody and a subsequent Western blot with an anti-Flag antibody demonstrated the zinc-dependent interaction between p-Smad2/3 and NFI-C. Furthermore, zinc deficiency in MDPC-23 cells rendered p-Smad2/3 unable to bind to NFI-C (Fig. 2B). These results suggest that zinc changes the binding efficiency between NFI-C and p-Smad2/3. Altogether, our data suggests that zinc is acceptable for regulation of expression and localization.



**Fig. 2. Subcellular localization of NFI-C in the presence or absence of zinc.**

(A) MDPC-23 cells were treated with 50 or 80 mM of zinc or 10 nM of TPEN. The cell lysates were then analyzed by Western blot using antibodies against NFI-C. Endogenous NFI -C was located in nucleus and cytoplasm, but NFI-C proteins were translocated by TPEN. N: nucleus, C: cytosol (B) 293T cells were transfected with both HA-tagged NFI-C and Flag-tagged Smad3 and treated with 80 mM zinc or 10 nM TPEN. The cell lysates were subjected to IP followed by Western blot with anti-HA and anti-Flag antibodies, or vice versa. In the presence of zinc, NFI-C interacted with p-Smad2/3.



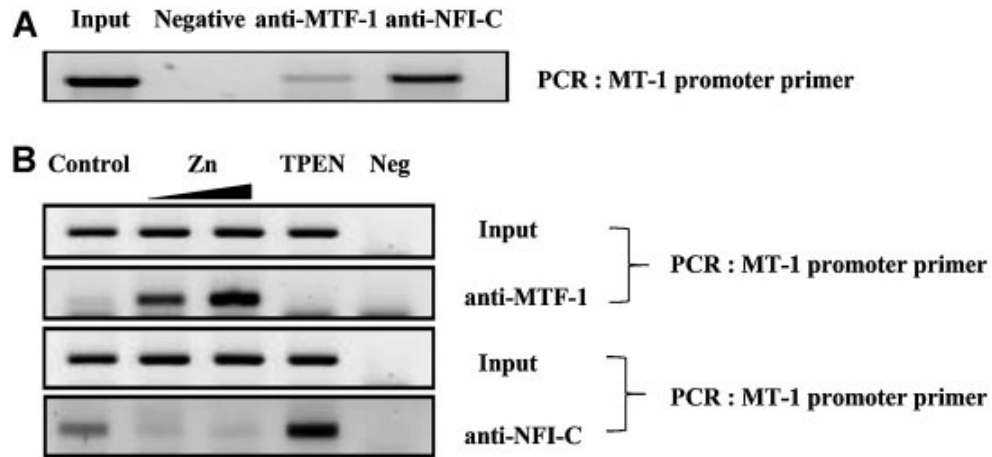
**Fig.S2. Expression of endogenous NFI-C protein in MDPC-23 cells in the presence or absence of zinc.**

NFI-C was immunostained in MDPC-23 cells with a rabbit polyclonal anti-NFI-C antibody and a fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (top panel). Exogenous NFI-C in MDPC-23 cells was detected by immunofluorescence after transfection of the GFP-tagged NFI-C construct (bottom panel). In the TPEN-treated group, NFI-C is primarily localized to the nucleus. Nuclei were stained with DAPI. Images were visualized by immunofluorescence microscopy.

### **3. Recruitment of NFI-C to the MT-1 promoter in response to zinc**

In a zinc-dependent manner, MTF-1 binds to DNA sequence motifs, such as metal response elements (MREs), with a core consensus of TGCRCNC in the promoter region (-200 to -30) of MT genes. Also, NFI is known as a transcription factor that binds overlapping MRE element sites in the mouse MT-1 promoter region and mediates MT-1 gene synergistically with MTF-1 [67]. To clarify the role of NFI-C and MTF-1, which could affect zinc responsive transcriptional activity by binding to MT genes in odontoblasts, we performed a ChIP assay for transcription factor binding properties. Chromatin DNA fragments were precipitated with the indicated antibodies and amplified using selective primers for binding sites in the MT-1 promoter region. The MT-1 promoter could be precipitated using an MTF-1 and NFI-C-specific antibodies. However, negative control (pre-immune serum) could not precipitate MT-1 promoter (Fig. 3A). In the zinc sufficiency condition, MTF-1 translocated into the nucleus, directly interacted with the MRE site on the MT-1 promoter (Fig. 3B), and eventually upregulated the MT-1 gene (Fig. 1A). In contrast, in the zinc deficiency condition, the recruitment of NFI-C to the MT-1 promoter was further increased (Fig. 3B), and the MT-1 gene was decreased (Fig. 1A). Consequently, MTF-1 and NFI-C can bind to the MRE site on the MT-1 promoter individually and can regulate MT-1 expression in response to zinc.





**Fig. 3. Recruitment of MTF-1 and NFI-C to chromatin**

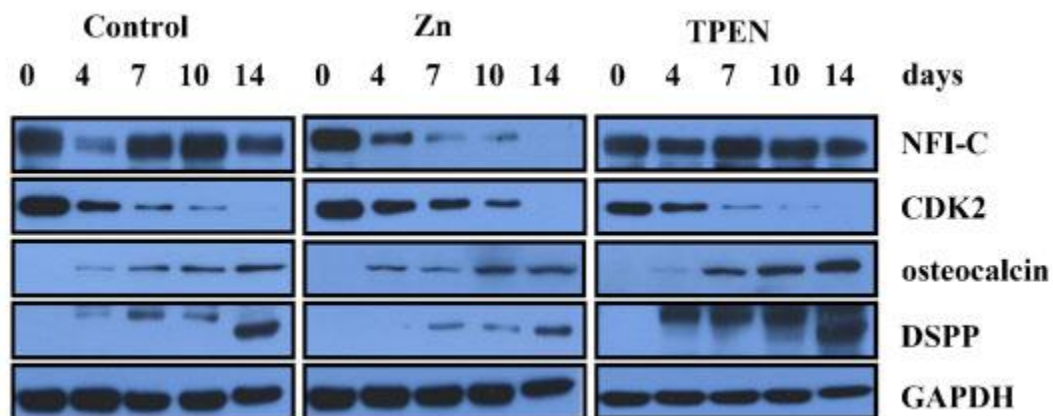
(A) Cross-linked chromatin was prepared and immunoprecipitated with pre-immune serum or MTF-1, NFI-C specific antibody. B: Cross-linked sheared chromatin was prepared from MDPC-23 cells treated with zinc (50 and 80 mM) or TPEN (10 nM) for 4 h. Chromatin samples were subjected to PCR analysis using primer pairs spanning the nearest MRE element site on MT-1 promoter. Zinc recruited MTF-1 and TPEN recruited NFI-C to the MT-1 promoter. The control represents the PCR product of chromatin obtained before IP with the MTF-1 and NFI-C antibodies. Neg: negative control.

#### **4. Zinc influences proliferation and differentiation**

In tooth development, odontoblasts differentiate in response to epithelial–mesenchymal interactions, which regulate proliferation, differentiation, and polarization [1]. Odontoblasts produce collagenous and non-collagenous proteins to form the dentin matrix [5]. Additionally, zinc is a structural element for DNA synthesis enzymes and is involved in regulating cell proliferation by second messenger mitogenic signaling [68]. MC3T3-E1 cells were increased in cell viability by additional zinc treatment of 15 mM for 1, 5, and 15 days [69]. However, the zinc-mediated cellular mechanisms in odontoblast cells have not been fully clarified.

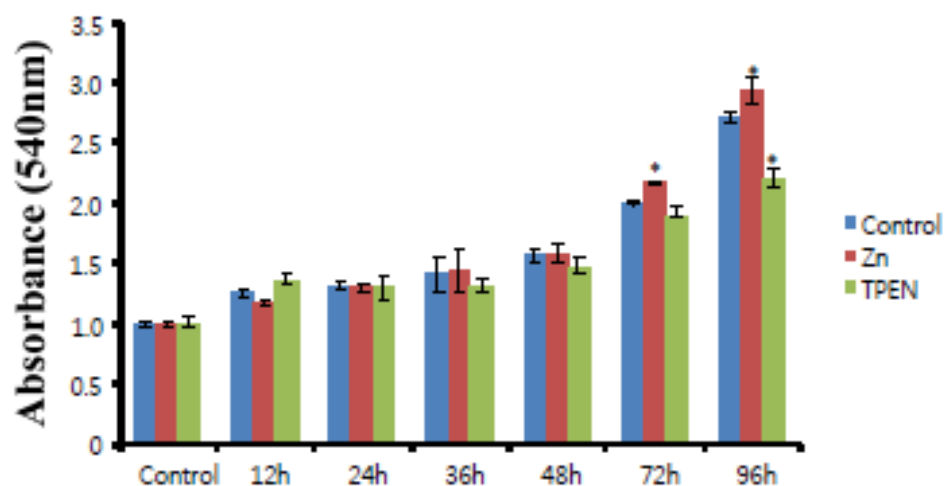
To evaluate the effect of zinc or TPEN on the proliferation and differentiation of odontoblasts, we treated MDPC-23 cells with zinc(10mM) or TPEN (1 nM) constantly in differentiated media and examined the protein levels of NFI-C, CDK2, osteocalcin, and DSP by Western blot analysis. The results showed that the NFI-C protein, a key transcription factor in odontoblasts, was expressed at the beginning of culture, was decreased at day 4, and increased again by day 10 in control cells. In the TPEN-treated group, NFI-C was strongly expressed throughout the entire culture period. In contrast, this protein decreased from the beginning of the culture to day 14 in the zinc-treated group. The level of CDK2, a cellular proliferation marker, was decreased from the beginning of the culture to day 10 in control, TPEN-, and zinc-treated groups. However, in zinc-treated group, although CDK2 protein was slightly decreased from the beginning of the culture to day 10, it was still clearly expressed by day 10 (Fig. 4). Moreover, we measured cell proliferation during early time points of differentiation by MTT assay. In

this result, additional zinc-treated group showed a slight increase in cell proliferation of early stage but TPEN-treated group did not show (supplemental Fig. S3). There were also notable differences in the expression levels of osteocalcin and DSP protein between the zinc and TPEN-treated groups. The expression of osteocalcin and DSP protein, a marker of differentiated odontoblasts, was significantly increased by TPEN treatment, but slightly decreased by zinc treatment (Fig. 4). These results suggest that zinc may maintain odontoblast proliferation properties during early differentiation stage, while zinc deficiency likely promotes odontoblast differentiation.



**Fig. 4. NFI-C, CDK2, osteocalcin, and DSP protein expression during odontoblast differentiation in vitro.**

Western blot analysis of NFI-C, CDK2, osteocalcin, and DSP expression during MDPC-23 cell differentiation in the presence of zinc (10 mM) or TPEN (1 nM). The cells were evaluated at 0, 4, 7, 10, and 14 days. NFI-C was strongly expressed in TPEN treated cultures.

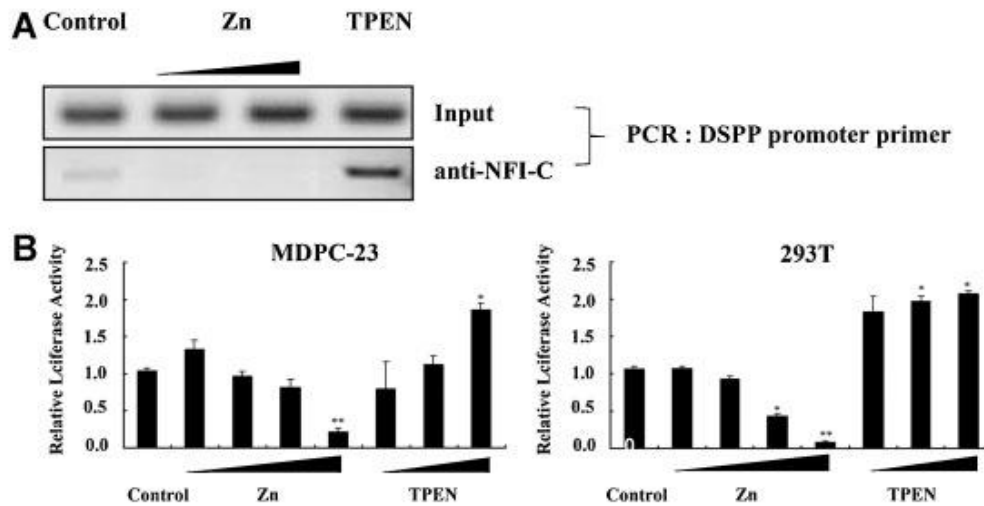


**Fig.S3. MDPC-23 cell proliferation analyzed by MTT assay**

MDPC-23 cells were untreated or treated with either zinc (10 $\mu$ M) or TPEN (1 nM) for 96 h and analyzed for proliferation by MTT assay as described in Materials and Methods. The data are presented as the mean  $\pm$  SD of triplicate experiments. Asterisks denote values significantly different from the control (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

## **5. Nuclear accumulation of NFI-C regulates DSPP transcriptional activation**

DSPP is secreted by odontoblasts and is involved in the dentin biomineralization process [5]. To elucidate whether the nuclear accumulation of NFI-C leads to transcription factor functionality, we next assessed DNA binding affinity and transcriptional activation. We performed a ChIP assay for the binding of NFI-C to the TTGGC (N5) GCCAA/CCAAT motif site on the DSPP promoter. In response to zinc, NFI-C was dissociated from the DSPP promoter. In contrast, zinc deficiency mediated by TPEN caused the recruitment of the NFI-C protein to the DSPP promoter (Fig. 5A). A second approach was taken to confirm the functional consequences of nuclear-accumulated NFI-C on DSPP promoter activity. Increasing concentrations of zinc significantly repressed the transcriptional activity of the DSPP promoter. However, TPEN enhanced its transcriptional activity in both MDPC-23 and 293Tcells (Fig. 5B). Therefore, it was suggested that DSPP might be upregulated through NFI-C binding, which is likely caused by zinc deprivation. Based on these results, nuclear NFI-C is biologically active for binding affinity and transcriptional activity in odontoblasts.



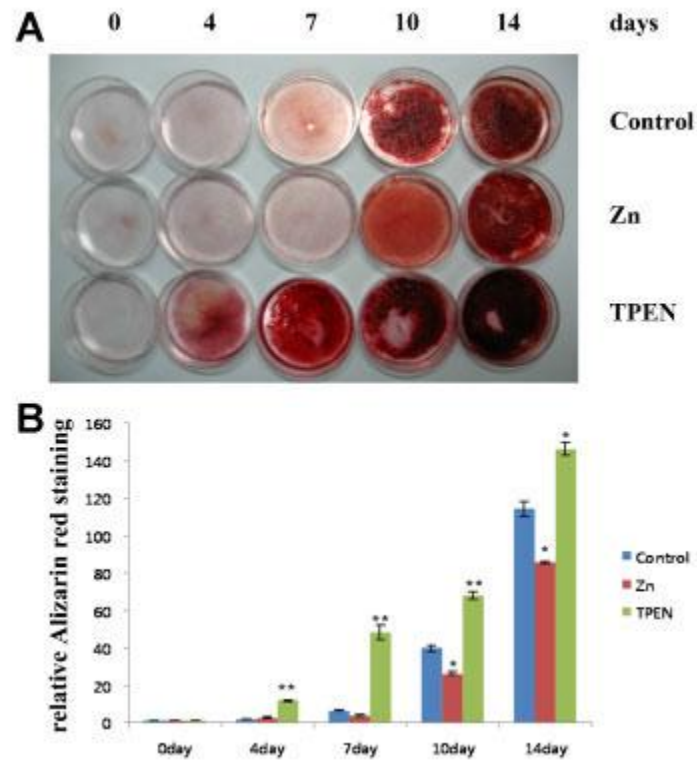
**Fig. 5. NFI-C functions as a transcription factor.**

(A) Cross-linked sheared chromatin was prepared from MDPC-23 cells treated with zinc (50 and 80 mM) or TPEN (10 nM) for 4 h. Chromatin samples were subjected to PCR analysis using primer pairs spanning the nearest TTGGC (N5) GCCAA/CCAAT motif site on the DSPP promoter. NFI-C was recruited by TPEN to the DSPP promoter. The control represents the PCR product of chromatin obtained before IP with the NFI-C antibody. (B) MDPC-23 and 293T cells were transfected with pGL3-DSPP. The cells were treated with increasing concentrations of zinc (10, 30, 50, and 80 mM) or of TPEN (1, 5, and 10 nM) for 4 h, and the lysates were then subjected to luciferase assays. The transcriptional activity of the DSPP promoter was inhibited by zinc in a dose-dependent manner, but was activated by TPEN. The data are presented as the mean  $\pm$  SD of triplicate experiments. Asterisks denote values significantly different from the control (\* $P$  < 0.05; \*\* $P$  < 0.01).

## **6. Effects of zinc on the mineralization of dentin in vitro**

Finally, we determined the effects of altered zinc concentrations on dentin mineralization. MDPC-23 cells were cultured for 14 days in differentiation media with continuous zinc or TPEN, and the mineralized nodules were evaluated by Alizarin red S staining. In normal MDPC-23 cells, mineralized nodules appeared after 10 days of culture. The zinc-treated group showed mineralized nodules after 10 days of culture in the same pattern as the control group. However, the number of mineralized nodules was slightly lower in the zinc-treated group compared to the control. Interestingly, the TPEN-treated group showed mineralized nodules after only 4 days, and the mineralized nodules increased with time during the culture period (Fig. 6A, B). These results suggest that zinc sufficiency can slightly delay dentin mineralization in vitro, while zinc deficiency initiates mineralization earlier and accelerates the formation of mineralized nodules.





**Fig. 6. Mineralization in MDPC-23 cells in the presence or absence of zinc**

(A) MDPC-23 cells were treated with 10 mM zinc or 1 nM TPEN for 14 days, and the mineralization was evaluated by Alizarin red S staining. B: Quantification of mineralization by colorimetric spectrophotometry. Odontoblast mineralization was altered by zinc administration. The data are presented as the mean $\pm$ SD of triplicate experiments. Asterisks denote values significantly different from the control (\*P < 0.05; \*\*P < 0.01).

## V. DISCUSSION

TGF- $\beta$ 1 overexpressing transgenic mice develop distinct dentin defects similar to those seen in *Nfic*-deficient mice [70]. Therefore, NFI-C and TGF- $\beta$ 1 are essential factors for both normal odontoblast differentiation and dentin formation.

Zinc plays important roles in TGF- $\beta$  signaling. Preconditioning with zinc stimulates the phosphorylation of a downstream factor, protein kinase C [71], and phosphorylated protein kinase C phosphorylates the MH1 domain of Smad3 and directly abrogates promoter DNA binding of Smad3 [72]. However, the functional relationship between zinc and NFI-C during odontoblast differentiation remains uncharacterized. In the present study, zinc stimulated the phosphorylation of Smad2/3 in cytoplasm and also induced the binding of p-Smad2/3 to the NFI-C protein. In contrast, TPEN treatment leads to NFI-C translocation to the nucleus. These results suggest that zinc may play a role in holding NFI-C in the cytoplasm by inducing the tight interaction between NFI-C and p-Smad2/3. Therefore, zinc could be a key element for mediating interactions between NFI-C and p-Smad2/3.

Differently spliced isoforms of NFI proteins (NFI-A, NFI-B, NFI-C, and NFI-X) play different roles in various tissues or with different promoters. The overexpression of NFI-C downregulates MT-1 promoter activity in HepG2 cells, whereas NFI-A and -B activate it [73]. In the present study, NFI-C interacted with the MT-1 promoter, and MT-1 was decreased in the zinc deficiency condition. We suggest that MT-1 gene expression in MDPC-23 cells is regulated by a transcriptional activator of MTF-1 and a transcriptional repressor of NFI-C, and this regulation is likely associated with different isoforms of NFI

proteins.

It was recently discovered that zinc and MT-1 induce DNA synthesis and mitogenic gene induction through transitioning the S-phase of cell cycle [74]. Also, MTF-1 is involved in tumor development, and the loss of MTF-1 results in the augmentation of ECM deposition [75]. In the present study, zinc sufficiency condition increased the expression of MT-1 and MTF-1, decreased NFI-C expression, and maintained the proliferation of MDPC-23 cells through the CDK2. In contrast, zinc deficiency condition reduced MT-1 and MTF-1 expression, and induced the differentiation of MDPC-23 cells through the upregulation of DSPP. These results suggest that the balance of zinc concentrations regulates odontoblast proliferation and differentiation.

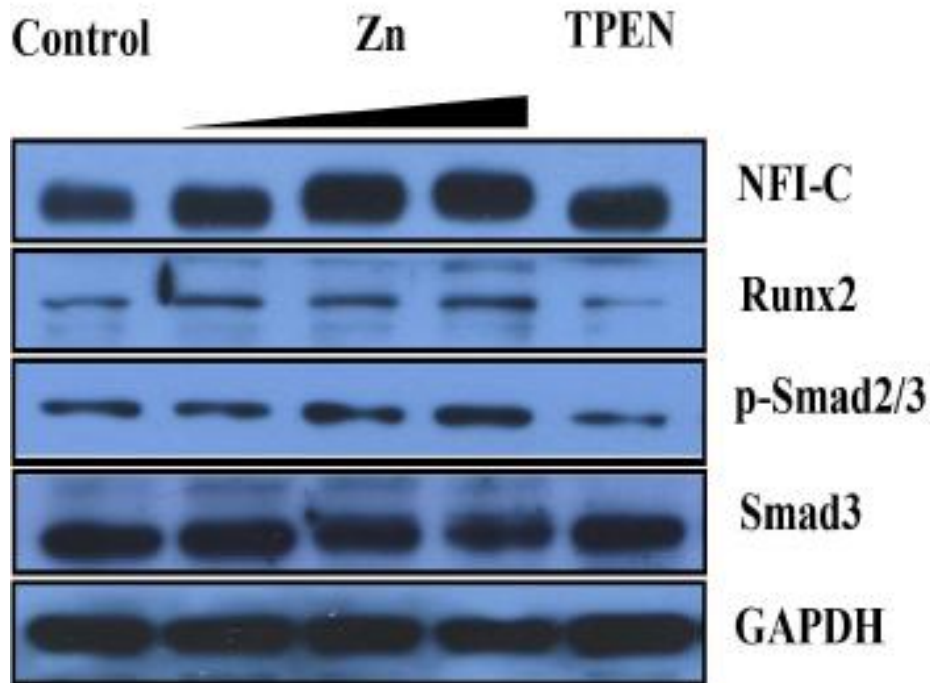
Mutations in the DSPP gene cause dentinogenesis imperfecta and dentin dysplasia [76, 77]. In addition, the DSPP gene promoter contains TATA and CAATT box sequences [78]. Transcription factors that recognize the CCAAT motif have been identified, such as CCAAT/enhancer-binding protein (C/EBP), CAAT-box transcription factor (CTF/NF-I), and CP1(NF-Y/CBF) [79]. Furthermore, C/EBP $\beta$  and NF-Y mediate DSPP expression and odontoblast differentiation [80, 81]. Thus far, there few reports indicate a direct interrelationship between NFI-C and DSPP promoters. However, in the present study, NFI-C interacted with the DSPP promoter, enhanced DSPP promoter activity, and, consequently, promoted the differentiation of odontoblasts and dentin mineralization. These observations suggest that NFI-C could mediate DSPP gene expression through recognized TTGGC (N5) GCCAA/CCAAT motifs.

Zinc stimulates bone formation by inducing the expression of osteoblast differentiation

marker genes, such as Runx2 and osteoprotegerin, and by inducing ALP activity and Ca deposition [56]. However, in the present study, zinc inhibited or delayed dentin formation. Also, zinc increased NFI-C in osteoblast-like MC3T3-E1 cells (supplemental Fig. S4), but decreased NFI-C in odontoblastic MDPC-23 cells. Zinc induces contrasting effects of NFI-C expression in osteoblasts and odontoblasts and regulates the transcriptional activity of the DSPP gene. It was reported that zinc-deficient diet mice decreased fetal long bone growth [82]. However, we could not find any tooth defects in these mice, including dentin (data not shown). The nutritional influence of zinc may not be sufficient to affect in dentin mineralization. Therefore, the relationship between zinc and NFI-C may act by different mechanisms between bone and dentin. Similar to our results, zinc plays a reciprocal role for calcification in bone (hard tissue) and blood vessels (soft tissue). In physiological process of bone formation, zinc enhances skeletal growth; however, in pathological process, zinc deficiency increases the calcification of smooth muscle cells, which is associated with plaque formation [56, 83, 84].

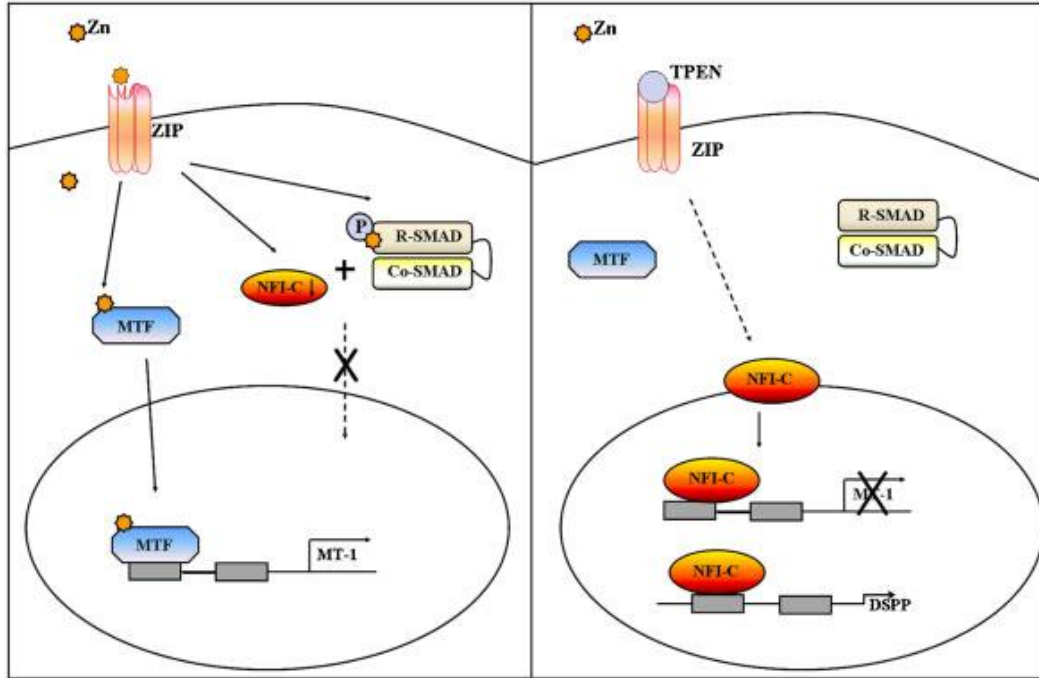
Zip13-KO mice showed reduced dentin formation of molar teeth in addition to reduced osteogenesis and abnormal cartilage development [23]. However, in the present study, zinc deficiency induced by TPEN promoted dentin mineralization in vitro. Based on these findings, we speculated that TPEN treatment resulted in the overall deficiency of zinc in the cytoplasm of odontoblasts, while Zip13-KO affects the distribution of zinc within the cytoplasm, causing a local zinc deficiency in the cytoplasm, especially near the Golgi complex. Recently, it was reported that dietary zinc-deficient mice up-regulate Zip13 mRNA expression [85]. This report has also provided evidence supporting our data.

As summarized in Figure 7, we arrived at several conclusions. The addition of zinc or TPEN to the culture media in MDPC-23 cells is associated with the induction of NFI-C expression, subcellular localization, and biological activity as a transcription factor.



**Fig.S4. Expression of NFI-C and Smad2/3 proteins in MC3T3-E1 cells in the presence or absence of zinc.**

MC3T3-E1 cells were treated with 30, 80, or 150  $\mu$ M of zinc or 10 nM of TPEN. The cell lysates were analyzed by western blot using antibodies against NFI-C, Runx2, p-Smad 2/3, Smad3, and GAPDH. Zinc caused the upregulation of NFI-C and Runx2, and it induced the phosphorylation of Smad2/3.



**Fig. 7. A crucial role for zinc in odontoblast biology (a mechanistic summary)**

Zinc promotes the binding of NFI-C and Smads in the cytoplasm, while the lack of zinc causes the NFI-C protein to function as a transcription factor in the nucleus. The balance of zinc is important for odontoblast differentiation and mineralization.

## **CHAPTER IV. CONCLUDING REMARKS**

The present thesis attempted to evaluate the role of odontoblast differentiation and dentin formation through epithelial-mesenchymal interaction. Specifically, we investigated biological function and mechanisms of Cpne7 and NFI-C in regulation of odontoblast differentiation and dentin formation.

During dentinogenesis, odontoblast differentiation was induced by epithelial-mesenchymal interactions. And Dentin is formed by odontoblasts which secrete extracellular matrix protein (ECM). Among ECM proteins, dentin sialophosphoprotein (DSPP) is the most abundant in dentin.

First, Cpne7 was expressed in preameloblasts and secreted extracellular during ameloblast differentiation. Also, the expression of Cpne7 showed a temporospatial distribution pattern during dentinogenesis in the developing crown and Cpne7 regulated odontoblast differentiation and dentin formation in vitro and in vivo. Moreover, Cpne7 induces differentiation of odontoblast-like cells from mesenchymal cells of dental or non dental origin. Cpne7 and nucleolin complex bound Dspp promoter and regulated Dspp gene expression. These results establish the role of Cpne7 as a key player in the regulation of mesenchymal stem cell differentiation into odontoblasts.

Second, NFI-C which was affected by zinc sufficiency condition may inhibit or delay the



differentiation and mineralization of odontoblasts; however, the nuclear accumulation of NFI-C following zinc deficiency could initiate earlier and accelerated differentiation through regulation of Dspp gene and mineralization of odontoblasts. Finally, zinc cellular equilibrium is extremely important for dentinogenesis.

Collectively, these studies provide the evidence for the Cpne7 and NFI-C effect and underlying mechanisms of odontoblast differentiation from undifferentiated mesenchymal cells and dentin formation.

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## CHAPTER VII. ABSTRACT IN KOREAN

### 상아모세포 분화와 상아질 형성 과정에서 CPNE7과 NFI-C의 역할

치아발생과정 중에서 상아질 형성은 상피간엽 상호작용을 통해서 이루어진다. 치유두의 외배엽성 미분화중간엽세포가 내치상피로부터 분화 유도작용을 받으면, 상아모세포로 분화한다. 이후 상아모세포는 유기질을 분비하고 이를 석회화 시켜 상아질을 형성한다.

상피간엽 상호작용을 포함한 상아모세포의 분화 유도와 관련된 연구가 일부 진행되었으나, 특정 유전자에 의한 정확한 생물학적 메커니즘에 대해서는 아직 명확히 밝혀진 바가 없다.

본 연구에서는 상아모세포의 분화와 상아질 형성 과정에서 치아상피 기원의 법랑모세포의 배양액에서 발견한 CPNE7 유전자와 상아질 형성과정에서 필수적인 인자로 알려진 NFI-C 유전자의 기능을 다양한 분자생물학적 실험을 통해 연구하였다.

조직학적으로 내치상피세포와 법랑모세포에서 CPNE7 단백질이 발현하였으며, 법랑모세포로부터 분비된 CPNE7 단백질은 상피간엽 상호작용을 통하여 상아모세포의 분화와 석회화를 촉진시켰다. 특히 상아모세포 내에서 CPNE7 단백질은 Nucleolin 단백질과 결합하여 상아질 특이 단백질인 DSPP 유전자를 직

접적으로 조절하였다. 또한 치수 재생 효과를 평가하기 위해 인간치수세포 (hDPCs)에 CPNE7 단백질을 처리하였을 때, 생리적인 치수조직이 잘 재생되었다.

Nuclear factor I (NFI) 전사인자는 4개의 그룹(NFI-A, NFI-B, NFI-C, NFI-X)을 가지고 있으며 여러 기관의 발생에 관여한다고 알려져 있다. 그 중 NFI-C 유전자가 없는 마우스의 경우, 치근이 비정상적으로 형성되고, 전치부도 부러지기 쉽게 변형되거나 이상이 발생하였다. NFI-C 유전자는 상아모세포 분화에 중요한 인자로써, 아연을 처리하였을 때, NFI-C 단백질은 세포질에서 smad2/3와 결합한다. 아연이 부족할 시 NFI-C 단백질은 핵 내로 이동하여 DSPP 유전자를 직접적으로 조절하여, 상아모세포의 분화와 석회화를 촉진 시킨다.

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주 요 어: CPNE7, NFI-C, 상아모세포 분화, 상아질 형성  
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